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PREFACE

This volume, although nominally equipped with a new editor and an additional associate editor, largely represents the planning of the previous, and original editor, Dr. D. I. Arnon. From its beginning under his guidance this series has become an established member of the Annual Review family and has made an ever increasing place for itself as a summarizer of progress in plant physiology. A few—the new editor must confess himself one of these—saw the establishment of this Review with some qualms, fearing that after one or two volumes, assembling all past progress, subsequent years would yield only small increments. These fears were quite obviously wrong; the well did not run dry, plant physiology became an ever more swollen stream, and approaches a torrent. The increasing pages of the review bear adequate witness to this fact—we hope without undue duplication on the part of the authors. Dr. Arnon's energy and confidence had a great deal to do with this success, for which all plant physiologists must be grateful.

It is a pleasure to announce that Professor A. S. Crafts will assume the editorship of this Review beginning with the next volume. From the interim editor to Dr. Crafts go the warmest of good wishes.

This volume presents one new feature—the welcome review by Dr. A. L. Kursanov on the present status of plant physiology in the U.S.S.R. We are extremely grateful to Dr. Kursanov for this article. It is clear that progress continues in the home of Palladin, Lubimenko, Kostychev, Maximov, and other leaders. Further reviews of this type may be possible in future years under a grant from the National Science Foundation.

Circumstances beyond the control of author or editors have prevented publication of the announced review on the Corn Plant. The reviews on Ascorbic Acid Metabolism, and on Plant-Soil Relations have, at the suggestion of the authors, been postponed to later issues.

Under the yearly rotation rules, Dr. W. M. Parker departs from the Editorial Committee, being succeeded by Dr. Sterling B. Hendricks of the U. S. Department of Agriculture. It is a pleasure to welcome Dr. Hendricks and to acknowledge Dr. Parker's long and valuable service on the Committee, which began with the first volume and extends to the planning of volume 8.

As always, the heavy burdens of editing, letter writing, and proof reading fall upon the editorial assistants of Annual Reviews. We are happy to acknowledge the devoted labors of Miss Beryl Daniels, Mrs. Marie Fahey, and Mrs. Michele Leiser in this connection. The subject indexing has been capably performed by Mrs. Grace MacNeill.

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TOPICS AND AUTHORS
ANNUAL REVIEW OF PLANT PHYSIOLOGY
VOLUME 8, 1957 (IN PREPARATION)

INTRODUCTORY CHAPTER (AUTOBIOGRAPHICAL), *W. J. V. Osterhout*
THE CONCEPT OF "APPARENT FREE SPACE" IN MINERAL NUTRITION, *G. E. Briggs and R. N. Robertson*
MINERAL NUTRITION (GENERAL), *H. G. Gauch*
PLANT-SOIL RELATIONSHIPS OF PHOSPHORUS, *L. A. Dean*
PLANT-SOIL RELATIONSHIPS (GENERAL), *R. L. Carolus*
NITROGEN METABOLISM, *F. C. Steward and J. K. Pollard*
PHOTOCHEMISTRY OF CHLOROPHYLL, *J. Rosenberg*
THE BIOLOGY OF PLASTIDS, *K. A. Clendenning*
SOLUBLE OXIDASES AND THEIR FUNCTION, *W. D. Bonner*
AUXIN RELATIONS IN ROOTS, *Börje Åberg*
METABOLISM OF AUXINS, *J. A. Bentley*
HERBICIDES, *W. B. Ennis, Jr.*
MORPHOGENESIS IN LOWER PLANTS, *L. Jaffe*
FLOWERING, *F. Lona*
STOCK AND SCION RELATIONS, *W. S. Rogers and A. Beryl Beakbane*
THE SUGAR CANE PLANT, *G. O. Burr*
MASS CULTURE OF ALGAE, *H. Tamiya*
PHYSIOLOGY OF PHLOEM, *K. Esau, H. B. Currier, and H. Cheadle*
PERMEABILITY, *R. Collander*
PHYSIOLOGY OF SALT TOLERANCE, *H. E. Hayward and L. Bernstein*
ASCENT OF SAP, *K. N. H. Greenidge*
DROUGHT RESISTANCE AND XEROPHYTISM, *W. S. Iljin*
PHYSIOLOGICAL ECOLOGY, *W. D. Billings*
PHYSIOLOGICAL GENETICS, *H. J. Teas*
ANTIBIOTICS IN HIGHER PLANTS, *P. W. Brian*

CONTENTS

MINERAL NUTRITION OF PLANTS: MECHANISMS OF UPTAKE AND TRANSPORT, *E. Epstein* 1

ENERGY TRANSFORMATIONS IN PHOTOSYNTHESIS, *L. N. M. Duysens* . 25

NITROGEN FIXATION BY PHOTOSYNTHETIC ORGANISMS, *G. E. Fogg* . . 51

ENDOGENOUS RHYTHMS IN PLANTS, *E. Bünning* 71

THE PHYSIOLOGY OF CELL DIVISION, *H. Stern* 91

OBLIGATE PARASITISM, *C. E. Yarwood* 115

BIOLOGICAL RELATIONS OF PLANT VIRUSES, *C. W. Bennett* 143

IRON CHLOROSIS, *J. C. Brown* 171

PHOTOPERIODISM IN WOODY PLANTS, *P. F. Wareing* 191

PLANT PROTEINS, *C. E. Danielson* 215

PHYSIOLOGY OF ROOT ELONGATION, *J. G. Torrey* 237

MECHANISMS OF CARBOHYDRATE BREAKDOWN IN PLANTS, *B. Axelrod and H. Beevers* 267

PHYSIOLOGY OF SEED GERMINATION, *E. H. Toole, S. B. Hendricks, H. A. Borthwick, and V. K. Toole* 299

PHOSPHORUS METABOLISM AND PHOTOSYNTHESIS, *D. I. Arnon* . . 325

ABSORPTION AND TRANSLOCATION OF PLANT REGULATORS, *J. van Overbeek* 355

EFFECTS OF LIGHT QUALITY ON PLANT GROWTH, *E. C. Wassink and J. A. J. Stolwijk* 373

RECENT ADVANCES IN PLANT PHYSIOLOGY IN THE U.S.S.R., *A. L. Kursanov* 401

INDEXES 437

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MINERAL NUTRITION OF PLANTS: MECHANISMS OF UPTAKE AND TRANSPORT[1,2]

By Emanuel Epstein[3]

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Regulation of the passage of solutes between cells and the solutions bathing them is one of the fundamental phenomena common to living things. Since inorganic nutrients participate in biochemical reactions as structural components of reactants, activators, and cofactors, the promiscuous presence of all kinds of ions, in all manner of concentrations and ratios, at the sites of all reactions, would preclude the orderly, integrated, and specific events which are in fact observed. A high adaptive value therefore attaches to the elaboration of structures and mechanisms which tend to protect the metabolic centers from the all pervading presence of the external solutes, and thus to create a relatively stable, regulated internal environment. Where the evidence indicates that the observed ion flux is a direct function of concomitant metabolism, we speak of active ion transport. Ion movements which are not active in this sense are common and are called passive, or, in the European literature, osmotic.

Several recent volumes summarize much work in this field, dealing, in many instances, with ion transport in animal tissues and microorganisms as well as in higher plants. *Active Transport and Secretion*, edited by Brown & Danielli (14), contains the papers read at a symposium of the Society for Experimental Biology. Five of the 27 papers deal with ion transport in plants. *Recent Developments in Cell Physiology*, edited by Kitching (60) is a collection of papers dealing with questions of transport as well as other topics of cellular physiology. There is evidence here of "transport" across conventional barriers of compartmentalization—a token of a trend in this field where the integration of diverse disciplines is vital [Hogeboom & Kuff (44)]. Clarke & Nachmansohn (21) have edited a volume entitled *Ion Transport across Membranes*. The title notwithstanding, only a few of the papers actually deal with this subject, the majority relating advances in

[1] The survey of the literature pertaining to this review was concluded in October, 1955.

[2] The following abbreviations will be used: ATP, adenosinetriphosphate; DNP, dinitrophenol.

[3] I wish to thank those of my colleagues who have made papers still in press available to me, and those who responded to my requests for reprints. Dr. S. B. Hendricks and Dr. H. A. Borthwick read the manuscript and made valuable suggestions, but are by no means accountable for the shortcomings of the paper. The Librarian, Helen C. Boyd and her assistant, Dorothy E. Kells, were indefatigable in their efforts to obtain materials for me.

protein and high polymer chemistry. None of these papers deal specifically with plants. *Electrolytes in Biological Systems*, edited by Shanes (100), is a collection of papers presented at a symposium of the Society of General Physiologists. These papers describe experimental work with plant and animal tissues as well as microorganisms.

Relevant topics recently or concurrently reviewed in *Annual Review of Plant Physiology* will be touched upon only in so far as new findings bear directly upon them. The reader is referred to Lundegårdh's (71) review dealing largely with his theory of anion respiration, to Williams's (123) article on redistribution of mineral elements during development, and to Pirson's (87) review of functional aspects in mineral nutrition. Closely related are the reviews on water relations by Kramer (62) and Walter (117).

PASSIVE ION MOVEMENTS

The absorption of ions by plant roots is often spoken of as an "active" or "metabolic" process. It has indeed been commonplace to say so, ever since the classical researches of Hoagland and his associates (42). Prolonged selective absorption of ions is inhibited by low oxygen tensions, low temperatures, and various poisons. These and many other criteria show it to be a manifestation of metabolic activity. However, work in recent years has renewed our interest in, and sharpened our perception of, passive modes of ion uptake, and has focused attention on their relation to absorption in the active sense.

Cation Exchange

Hanson & Bonner (39), using Jerusalem artichoke tuber tissue, found that there was a rapid uptake of rubidium during the initial hour, followed by a phase of slower absorption lasting many hours. These findings confirmed earlier observations by Steward & Harrison (109) on the uptake of rubidium by tuberous tissue of potato. The initial rapid uptake was identified as cation exchange. When uptake was followed by measurement of changes in the conductivity of the external solution, the initial rapid phase was not observed, the cations displaced from the root by rubidium ions taking the place of the latter in the solution, with no change in conductivity. Epstein & Leggett (33) observed a similar two-component time course in the absorption of labeled strontium by excised barley roots. Labeled strontium taken up by cation exchange could subsequently be displaced from the exchange spots by unlabeled strontium, and, equally effectively, by calcium or magnesium ions. The monovalent cations, potassium and sodium, were less effective than the alkaline earths in displacing exchangeable strontium, potassium having a slightly greater affinity for the exchange spots than did sodium. In other words, the roots acted as cation exchangers, like clays or synthetic cation exchange resins, and exhibited the lyotropic series of affinities common in such systems. Higinbotham & Hanson (41) found that the initial, rapid phase of rubidium uptake by potato tuber tissue was related to the external

concentration by the Freundlich adsorption equation. Labeled rubidium taken up during the initial phase was exchangeable with unlabeled rubidium in the external solution. Sutcliffe (113, 114) observed fractions of exchangeable potassium in red beet root tissue. Massart & van der Stock (73), Massart (72), and Sherman (104) have discussed cation exchange in microorganisms, and Anscheutz & Gessner (1) in *Sphagnum* moss.

The occurrence of cation exchange in plant tissues is thus well attested in many investigations, and for both storage tissue and fibrous roots of higher plants. Uptake by this mechanism has frequently been termed "passive," because it occurs even when the tissue is exposed to conditions which minimize metabolic activity and interfere with the prolonged, steady absorption which is usually observed subsequent to the rapid, initial uptake. Russell (95) has criticized the use of the terms "passive" and "physical" as applied to uptake by ion exchanges, since these processes depend on metabolically produced substances, "equally with the active mechanism of accumulation." This criticism beclouds a useful and, indeed, necessary distinction. If ion exchange occurs in roots, the process, of course, depends on metabolically produced substances; in other words, it depends on the existence of the roots, but this does not make the process of ion exchange itself an active one. Even dead roots exhibit cation exchange [Jacobson & Overstreet (58); Rediske & Selders (88)], but one would hardly hesitate to call this a passive process, although even the dead roots' existence depends on prior metabolism. Unlike the process of active absorption, passive ion exchanges do not require concomitant metabolic activity on the part of the tissue. Poisons may, however, diminish the number of exchange spots available in the tissue, and thereby decrease the extent to which ion exchange occurs [Russell & Ayland (96)]. On the other hand, 2,4-dichlorophenoxyacetic acid (2,4-D) may cause growth, thus providing additional exchange spots and increasing the cation exchange capacity of the tissue [Hanson & Bonner (39)]. The evidence being conclusive as to the occurrence of cation exchange, the question arises whether or not it is an integral part of the train of events constituting the absorption process. Epstein & Leggett (33) showed that the two processes, cation exchange and active transport, differ in attributes relating to kinetics, reversibility, ion specificity, and energy requirements. It would appear on this evidence that the exchange surfaces or spots are not identical with the entities involved in active transport, but the question still remains whether an initial exchange on the exchange surfaces or spots is a step necessarily preceding the entry of the ion into the active transport mechanism. Higinbotham & Hanson (41) leave this question open. Russell (95) seems to answer it in the affirmative, indicating (p. 362) that cations initially exchange for hydrogen ions, this step being followed by transport mediated by the carrier. Epstein & Leggett (33), on the other hand, conclude that an adsorption on the cation exchange surfaces is not a rate-limiting step in strontium absorption, since displacement of strontium from the exchange surfaces by other cations bears no consistent relation to

the effect of these cations on the rate of active strontium absorption. In fact, since there is constant isotopic ion exchange between the exchange surfaces and the solution, it often becomes a moot question whether or not an ion travels to the carrier sites over the exchange surfaces or in solution (including solution within the tissue—see below). The two phases are in equilibrium.

However, the amount of a given cation adsorbed on the exchange spots can be minimized if another cation is present in excess, preferably one with a greater affinity for the exchange spots than that of the ion whose rate of absorption is under consideration. Viets (126) and Overstreet, Jacobson, & Handley (85) measured potassium absorption by excised barley roots in the presence of a great excess of calcium. Calcium has a greater affinity for the root exchange surfaces than the monovalent potassium [Epstein & Leggett (33)]; and, hence, under conditions of a high Ca/K ratio, will largely displace potassium from the exchange spots. If absorption by the active mechanism were contingent upon an initial adsorption on the exchange surfaces, displacement of potassium from the exchange surfaces by calcium would be expected to result in a reduction in potassium absorption. But this expectation is not borne out by the experimental evidence. This result fails to support the view that an initial exchange reaction is a necessary first step in cation absorption. The effects of sodium and lithium on the rate of rubidium absorption, observed by Epstein & Hagen (32), also argue against a rate-limiting role of exchange adsorption in active absorption. It is of interest here that Charles (20) concluded that movement of basic dyes and antibiotics in petioles was retarded by their adsorption on negatively charged cell surfaces. Anionic substances moved freely.

Reichenberg & Sutcliffe (89), Vervelde (125), and Sollner (107) have presented theoretical discussions of exchanges of both cations and anions in amphoteric systems. However, at normal external pH values, the acid dissociation predominates [Hope (49)], so that adsorptive ion exchange in plant roots largely is cation exchange. Before leaving this subject, a remark on terminology is in order. There has been a plethora of terms to describe what we have called "exchange surfaces" of the root, or "exchange spots." Hope (49) speaks of "nonmobile ions" in the cytoplasmic phase, Lundegårdh (70) of "adsorption tracks," Hanson & Bonner (39) of the "cation exchange capacity" of the tissue, Hylmö (54) of "electrically charged points" in the cell wall and cytoplasm, and Frey-Wyssling (35) of "immobile anions." Nevertheless, there seems to be substantial agreement as to the meaning of these terms, viz., fixed or at least constrained negative valencies to which cations are attracted by Coulomb forces. The chemical nature of the compounds bearing these valencies is unknown, and it is likely that a number of different substances contribute to the total cation exchange capacity. Two of the terms used may not be entirely fortunate. Epstein & Leggett's (33) "exchange surfaces" of the root might be misconstrued as a reference to the geometric surface of the root. The term was used in the sense in which it is often used in clay chemistry [Stout & Overstreet (111)], en-

compassing the sum total of all surfaces available for exchange, "internal" as well as "external." Lundegårdh's (70) term, "adsorption tracks," implies that exchange spots are lined up in the direction of net ion movement, but there is no evidence to that effect.

Diffusion

Conway & Downey (25) showed that succinic acid freely entered a space within the yeast cell which was equal to about one-tenth the total cell volume. Neither inulin, peptone, nor divalent succinate ions had access to this region. The measured volume did not involve the spaces between the packed cells, which were accounted for separately. It was an intracellular space, and Conway & Downey called it the "outer region" or "space" of the cell. This space was freely acessible not only to a great variety of organic acids, but also to neutral substances (sugars) and inorganic ions. Cowie, Roberts & Roberts (27) found that the entire "water space" (equaling the total cell water) of *Escherichia coli* was rapidly and reversibly acessible to potassium and sodium ions. Equality of concentration with the external solution was reached in less than five minutes. Entry of the ions into, and exit from, the water space was not a function of metabolism. Roberts, Abelson, Cowie, Bolton, & Britten (91) using the same organism, found the water space accessible to sulfate, phosphate, manganese, glucose-1-phosphate, fructose-1,6-diphosphate, and amino acids. Similar findings are being reported for animal tissues. Thus Whittam & Davies (121) found an "outer space" in guinea pig kidney-cortex tissue which was freely accessible to sodium ions. On the assumption that the concentration of the ion in this space equaled its concentration in the medium, the "outer space" occupied approximately 50 per cent of the tissue at 37°C., suggesting that the barrier to the exchange of the rest of the cell sodium is not the outer cell membrane but may lie within the cell. Shanes & Berman have studied diffusion in the toad sciatic nerve (101, 102) and the squid giant axon (103).

Hope & Stevens (51) were the first to make measurements of regions accessible by diffusion in plant roots and called this region the "apparent free space" (A.F.S.) of the tissue. Hope's (48) earlier work on membrane potential differences in bean roots had suggested that inorganic ions diffuse through or into the protoplasmic membrane. Following this clue, Hope & Stevens (51) found, by means of conductivity measurements on the external solution, that there is a reversible diffusion of KCl into bean root tips, equilibrium being attained in about twenty minutes. This process was clearly distinguished from absorption in the sense of active, metabolic transport. On the assumption that at equilibrium, the concentration of KCl in the "apparent free space" equaled its concentration in the external medium, the space accessible by diffusion was equivalent to 13 per cent of the weight of the tissue. Most of this space was believed to be protoplasm, as neither intercellular spaces nor the cell wall lattice could account for more than a total of about 3 per cent of the tissue. In a subsequent paper, Hope (49)

found that the measured "space" increased with an increase in the concentration of KCl used in measuring it. The findings indicated the existence of a Donnan distribution of the diffusible ions, resulting from the presence of nonmobile anions or cation exchange spots in the root cell cytoplasm. Butler (18) measured the "apparent free space" in wheat roots and found values ranging from 25 per cent to 34 per cent of the tissue. He used chloride, phosphate, and mannitol. Hylmö's (53) value for pea roots was 7 to 8 per cent, using chloride as the diffusing ion. The value may be too low, since, as the author pointed out, the inner parts of the thick roots may not have equilibrated in the experimental period.

Measurements of the "apparent free space" are ambiguous in that the "space," expressed as a volume, represents, partly, a real volume and, in part, a contribution which may be positive or negative, resulting from the presence of cation exchange spots in the tissue. These valencies result in a Donnan distribution of the ions [Hope, (49)]. Epstein (31) separately accounted for ions held by labile binding or exchange effects and measured, in barley roots, an "outer" space of approximately 23 per cent of the volume of the tissue. The space was constant over a wide range of concentrations of sulfate used in measuring it. It did not vary over the pH range of 4.0 to 7.7, and was essentially the same whether measured by means of sulfate, selenate, phosphate, or calcium ions. Epstein (31) demonstrated removal of labeled sulfate from this "outer" space of the tissue through absorption by the active transport mechanism, with no change in total labeled sulfate in the tissue. He concluded that the immediate substrate for absorption by the active, metabolic mechanism is the solution in the "outer" space of the tissue, this solution in turn tending toward equilibrium with the external solution.

The above findings all indicate that many types of cells and tissues contain appreciable spaces which tend to be in equilibrium with the solutions bathing them, so far as inorganic ions, and certain other solutes as well, are concerned. Penetration of ions into these "free" or "outer" spaces is reversible and nonmetabolic, but may be a prerequisite to subsequent active, metabolic transport. The magnitude of the spaces measured and the considerations presented by Hope & Stevens (51) and Hope & Roberston (50) strongly suggest that these spaces, in plant roots, are not extracellular (i.e., intercellular spaces) but involve the cell cytoplasm. Höfler (43), from other evidence, has reached a similar conclusion for angiosperms, though not for algae, especially desmids. In the angiosperms tested, sodium carbonate penetrated readily through the cytoplasm, as far as the tonoplast. Brooks (10) reported similar findings even for algae, in experiments on sodium penetration into the cytoplasm of *Valonia* and *Halicystis*. Certainly, identification of this space with the cytoplasm is inescapable in the case of *Escherichia coli*, where the findings of Cowie & Roberts (26) indicate that most or all of the "water space" (which equals the total cell water) is freely and reversibly accessible to inorganic ions, sugars, sugar phosphates, and amino

acids. In other words, in many kinds of cells and tissues where appreciable "free" or "outer" spaces have been observed, the outer surface of the protoplasts does not seem to offer any great resistance to the passage of those substances to which the measured space is accessible. Many of these substances are required in metabolism as activators, cofactors, and intermediates, and the question arises why they do not normally diffuse out and become lost to metabolism. Cowie & Roberts (26) conclude that while free metabolites in this space can indeed diffuse out, metabolites involved in the metabolic reactions of the cell are not free but attached to some larger molecules, and thereby prevented from diffusing out. It is of interest in this connection that recent findings indicate loss of amino acids from plant roots [Katznelson, Rovatt & Payne (59); Parkinson (86); Linskens & Knapp (67)]. Arisz (6) reports the remarkable finding that in the leaves of *Vallisneria* there occurs a light dependent, reciprocal movement of chloride between the vacuole and cytoplasm, without any loss to the outer solution. On the basis of this finding and from experiments with inhibitors, Arisz (4) concluded that in this tissue there is an accumulating mechanism near the outer boundary of the cytoplasm in addition to the one near the tonoplast. Alternatively, the ions in the cytoplasm may be bound to larger molecules, according to the concept discussed by Cowie & Roberts (26).

ACTIVE TRANSPORT

Diffusion of ions in "free" or "outer" spaces and exchange of cations on electronegative spots are passive, reversible processes. Diffusion entails no binding at all, by definition, and cations constrained by fixed electronegative valencies are readily displaced by other cations in the solution, a process characterized by the usual lyotropic series of affinities. The active, metabolic mechanism of absorption exhibits an entirely different and more specific selectivity. Both diffusion and cation exchange result in equilibrium within a short time after exposure of the tissue to a new solution but active absorption may proceed for long periods, at steady rates.

Carriers

There appears to be wide agreement now that active transport of ions in plant roots involves the operation of "carriers." Among recent writers, only Vervelde (125) seems to consider a carrier theory inacceptable. The essential features of the functioning of carriers are the attachment of the ions to carrier molecules, the movement of the resulting carrier-ion complexes through some barrier intervening between an "outer" and an "inner" phase, and the subsequent discharge of the ions into the "inner" phase. The first reference to such a system in plant roots (sugar cane) seems to be that in a paper by van den Honert (46) who compared the absorption mechanism with a constantly rotating belt conveyer. Other pioneering statements are by Osterhout (83) and Brooks (9), both of whom worked with algae, and by Wohl & James (124) in a theoretical paper not referring to any particular

type of organism or tissue. The carrier concept rests on a number of different kinds of observations, each of which, taken singly, would be fairly persuasive. Taken together, they make a strong case.

Isotopic exchange.—Part of the evidence comes from exchange studies, and in this connection radioactive isotopes have been particularly valuable. It has repeatedly been found that the fraction of absorbed ions whose uptake was mediated by the active, metabolic mechanism is largely nonexchangeable with other ions of the same ionic species. Since not only back diffusion, but even isotopic exchange of this fraction is largely prevented, the barrier responsible for the retention must be considered highly impermeable to the ions in question. Nevertheless, the ions are absorbed, and this leads to the conclusion that they pass through the barrier not in the form of free ions, but in combination with carriers. Once the ions dissociate from the carriers at the inner surface of the barrier, they are prevented from diffusing back by the impermeable barrier. This aspect of the problem was lucidly discussed by Osterhout (84). Sutcliffe (113) studied the exchangeability of monovalent ions in cells of red beet root tissue. He wanted to test the hypothesis, among other things, that the measured net absorption might be the result of accumulation on the one hand and leakage on the other, since he failed to find the constant relationship between "anion respiration" and metabolic uptake demanded by the Lundegårdh theory. He found that neither potassium nor bromide exchanged readily, and concluded that active transport of these ions involved their passage, in combination with specific carriers, across a barrier highly impermeable to the free ions. Plasmolysis induced leakage of absorbed ions, and interfered with their metabolic absorption as well [Sutcliffe (112)]. Epstein & Leggett (33) found that labeled strontium taken up by the respiration-dependent, active mechanism of barley roots was nonexchangeable with unlabeled strontium in the external solution. Similarly, labeled sulfate absorbed by the active transport mechanism was not lost to any appreciable extent upon transfer of the roots to solutions of unlabeled sulfate [Epstein (31)]. In both the strontium and sulfate studies large reversible fractions of the ions were observed, but their uptake was nonmetabolic and accounted for by cation exchange and diffusion in an "outer" space of the tissue, as discussed above. The occurrence of such large, passive ion permeations permits the conclusion that the barrier preventing back diffusion and isotopic exchange of the metabolically absorbed ions in the "inner spaces" cannot be identified with the epidermis.

Many studies, then, indicate a far reaching nonexchangeability of actively transported (absorbed) ions in a variety of plant tissues. Oddly at variance with these rather consistent findings are those of Scott & Hayward (97) for the green alga, *Ulva lactuca*. All of the potassium in the cells was exchangeable. Increases in temperature from 20° to 30°C. and light each favored a more rapid attainment of equilibrium. The exchange is reminiscent of tissues of animal rather than plant origin.

Concentration effects.—Another line of evidence concerning ion transport

by carriers comes from studies of the effect of external concentration on the rate of absorption. Numerous findings indicate that at increasingly higher concentrations a limiting rate of absorption is asymptotically approached. These findings suggest that some finite entity is occupied progressively as the external concentrations increase, a maximal rate resulting from complete saturation of this entity. This cannot, however, represent an equilibrium condition, as in clays or synthetic exchange resins, because a given rate of absorption, corresponding to a certain external concentration, may be maintained for long periods. These considerations and findings suggest a turnover mechanism whereby the ion binding entity discharges the ion again into a compartment other than the external solution; in other words, into "inner" spaces of the cells. Thus the evidence from the effects of concentration on the rate of absorption again leads to a carrier scheme. Considerations of this type form the basis for the enzyme-kinetic hypothesis of ion absorption by Epstein & Hagen (32), extended in later papers. In this view ion transport mediated by carriers is compared with enzymatic catalysis. In each, a substrate to be acted upon (the enzyme substrate or "substrate ion," respectively) is attached to an active agent (enzyme or carrier) and, upon completion of the process in question (catalysis or transport), the intermediate complex breaks down again. This last step is the rate limiting reaction, and is essentially irreversible. Rate measurements consistent with this hypothesis have been reported for alkali cations [Epstein & Hagen (32)], halide [Epstein (28)], alkaline earths [Epstein & Leggett (33)], sulfate [Leggett & Epstein (64)], and orthophosphate [Hagen & Hopkins (38)]. Rosenberg and Wilbrandt (93a) have recently given a theoretical treatment of the kinetics of membrane transports involving chemical reactions.

In the experimental papers quoted, the relation between concentration of an ion and its rate of absorption is that of a Langmuir isotherm. Upon plotting the reciprocals of these variables, straight lines are obtained according to Lineweaver & Burk (66). Higinbotham & Hanson (41) examined the relation between the external rubidium concentration and rubidium absorption by excised potato tuber tissue and found that both the initial, rapid phase of uptake and the prolonged accumulation against a concentration gradient followed the relation of a Freundlich adsorption process in their dependence on the external concentration. After several hours, however, the rate became independent of the external concentration except at the lowest concentrations, indicating that the tissue reached a level of uptake (in terms of absolute amounts) at which factors other than the external concentration became rate limiting. Such factors apparently were involved in the experiments of Olsen (79, 80) in which there seemed to be no concentration dependence over wide ranges of external ion concentrations. By way of contrast, Knauss & Porter (61) reported that absorption of calcium, strontium, iron, manganese, zinc, and copper by *Chlorella pyrenoidosa* was directly proportional to the concentration of the ions in the nutrient solution over remarkably wide ranges of concentration. Only with phosphorus and sulfur were lim-

iting values approached at high concentrations. It seems likely, however, that the same would hold with the other elements studied if still higher concentrations were used. For copper, there is in fact a suggestion of a leveling off at the highest concentration used.

It is clear from these several findings that no one simple equation invariably describes the relation between the concentration of an ion and the rate of its absorption. The plant material used, its initial nutritional status, the ranges of concentration explored, the duration of the experiments, and other experimental conditions are all factors to be considered. It is essential to make sure in kinetic studies of active transport that rates of metabolic absorption are measured, rather than heterogeneous, lump values of uptake. Russell & Ayland (96) failed to find the relation between concentration and uptake discussed by Epstein and co-workers [28, 32, 33, 64)]. Russell & Ayland worked with storage tissue, like Higinbotham & Hanson (41), but unlike these workers and Epstein & Leggett (33), they failed to distinguish between the initial exchange equilibration and steady-state active transport. The former represents a very large percentage of the total uptake by storage tissue from solutions at low concentrations and for relatively short experimental periods. Thus it must be concluded that theoretical interpretations based on such data as Russell & Ayland's are likely to be misleading until the steps in the overall process of ionic absorption are experimentally sorted out. On similar grounds, Honda (45) has questioned Russell's (95) conclusion that ascorbic oxidase supports salt accumulation by barley roots. The uptake measured by Russell may have been by mechanisms other than active transport.

Mutual ion effects; specificity.—In addition to isotopic exchange studies and experiments on concentration effects, examination of the mutual interference of ions in the absorption process has provided evidence concerning transport mediated by carriers. If in the process of transport, ions are attached to carriers, as postulated, this binding would be expected to provide the chemical basis for the selectivity which is in fact observed. As discussed above, the exchange and diffusion equilibration which takes place upon transfer of tissue to a new solution cannot account for the specificities observed in steady-state, active absorption. Epstein & Hagen (32) found in short-term experiments with excised barley roots that potassium and cesium specifically competed with rubidium, but sodium (except at high concentration of either sodium or rubidium) and lithium did not compete with rubidium. The halides were found to be competitive, but nitrate had no affinity for the halide binding sites [Epstein (28)]. Calcium and barium competed with strontium for binding sites for which magnesium had but little affinity [Epstein & Leggett (33)]. Selenate competed with sulfate, but neither nitrate nor phosphate competed for the binding sites common to those two ions [Leggett & Epstein (64)]. The observed specificities are concordant with earlier observations on ion selectivity and "antagonism" in higher plants, as discussed by Epstein (30). The general conclusion from

these studies is that ions recognized as being similar in chemical behavior tend to act as metabolic analogs in the absorption process and to compete for identical sites on the carriers effecting active ion transport. Burström (16) has misinterpreted these specificities as applying to the initial exchange and diffusion phase of uptake. On the contrary, this selective binding is a characteristic of steady-state, active transport. Diffusion, by definition, entails no selectivity, and the selectivity exhibited by the initial adsorptive cation exchange is quite different from the specificity which characterizes the active transport mechanism [Epstein (28); Epstein & Leggett (33)].

Menzel & Heald (75) studied the absorption and distribution of potassium, rubidium, and cesium, and of calcium and strontium, in millet, oats, buckwheat, sweet clover, and sunflower grown in nutrient solutions. There was a fixed relation between the ratio of rubidium to potassium in the solution and the ratio of these elements absorbed by the plants. Absorption of rubidium was 0.85 times that of potassium; for cesium, the factor was 0.20. Strontium was favored in absorption over calcium by a factor of 1.1. These factors were nearly constant for all the ratios of the ions in the nutrient solutions that were tried, and differences between plant species were not significant. However, the ratios of the several elements in various parts of the shoot differed significantly from the ratios obtaining for absorption by the plant as a whole. Thus, although these findings are consistent with the view of competition between potassium, rubidium, and cesium in the absorption mechanism, and between calcium and strontium, they nevertheless give reason for caution in the use of Rb^{86} as a tracer for potassium, or of Sr^{89} to label calcium. Shrift (105) concluded from experiments with different sulfate/selenate ratios in the medium that these two ions competed in the absorption process in *Chlorella vulgaris*, as well as for enzyme sites involved in their subsequent metabolism within the cell. Similar factors seemed to operate in the absorption and metabolism of L-methionine and its selenium analogue [Shrift (106)]. Competitive antagonism between sulfate and selenate, and methionine and seleno-methionine, has also been demonstrated in *Aspergillus niger* [Weissman & Trelease (120)]. In general, specificites like those observed in connection with the transport of inorganic ions may be met with in the transport of organic substances as well [Arisz (3); Rothstein (94)], and selective binding by carriers or enzymes has been invoked to account for transport of these substances, as with inorganic ions. In the green alga, *Ulva lactuca*, two independent metabolic mechanisms are operative in regulating cation fluxes; one for moving potassium inwards and the other for transporting sodium outwards [Scott & Hayward (98, 99)]. The reciprocal movements of potassium and sodium are reminiscent of those occurring in yeast [Conway (23)] and muscle fibers [Steinbach (108)]. A special case of selectivity is reported by Hagen & Hopkins (38). The absorption of orthophosphate by excised barley roots was studied as a function of the phosphate concentration of the external solution and its pH. Kinetic analysis of the findings resulted in the conclusion that the two ionic species, $H_2PO_4^-$ and

$HPO_4^=$, were bound by different carrier sites, and that hydroxyl ions competed at both positions.

Effects of one ion on the absorption of another ion of like sign are not necessarily inhibitory or "antagonistic." In some instances, promoting or "synergistic" effects have been observed. One of the most interesting of these is the promotion of the absorption of monovalent ions by calcium and other di- and polyvalent cations [Viets (126); Overstreet, Jacobson, & Handley (85)]. Fawzy, Overstreet, & Jacobson (34) have examined the absorption of potassium by excised barley roots as influenced by preceding or concurrent exposure to various monovalent, divalent, and trivalent cations at pH values ranging from 1 to 6. The negative effects of low pH values on retention and absorption of potassium were counteracted by monovalent, and much more effectively, by calcium and trivalent cations. At the usual physiological pH values, calcium and certain trivalent cations increased potassium absorption over that of the controls (KCl only). The data are interpreted as indicating a promotion, by the effective cations, of a reversible reaction between potassium ions and the hydrogen form of a carrier, favoring the formation of the carrier-potassium complex. Burström (17) found that calcium was indispensable for the absorption of nitrate by wheat seedlings and considered that calcium may be necessary for the formation of the carrier involved in nitrate absorption. Tanada (116) has investigated the effects of calcium and ultraviolet radiation on the absorption of rubidium and phosphate by excised mung bean roots. Calcium markedly stimulated the absorption of both ions. Irradiation increased the absorption of rubidium in the absence of calcium, but in the presence of calcium, rubidium absorption of the irradiated tissue was much reduced. Phosphate absorption was diminished by irradiation both in the absence, and somewhat more severely, in the presence of calcium. The results are interpreted as suggesting the involvement of ribonucleoproteins as ion binding carriers in salt absorption. Leggett & Epstein (64) showed that the rate of absorption of a divalent anion (sulfate at pH4) was increased by calcium.

Metabolism and Biochemistry

The preceding discussion has emphasized that most workers in the field of ion transport look upon the phenomena investigated, not in terms of the properties of structures ("permeability," "exchange capacity"), but rather in terms of the activities of agents ("carriers"). This is not to deny that the integrity of the structural framework of the cells and of subcellular entities is essential to the overall process, but to emphasize that the existence of this framework in itself will not result in ion transport. Only the diffusion and exchange equilibration discussed above under Passive Ion Movements will proceed relatively unaffected under conditions of arrested metabolic activity. Such considerations emphasize the importance of studies dealing with the effects of metabolic factors on ion transport, and conversely, the effect of ions on the course of metabolism. It is important, in this connection,

to consider carefully the effects of the experimental conditions, since exposure of tissues to a given ion may cause growth responses which in turn might modify the structures and mechanisms involved in the absorption of that ion [Hassan & Overstreet (40); Libbert (65); Miller (76)].

Respiration.—Since all energy consuming processes of metabolism ultimately depend on respiration, the relation between ion absorption and metabolism is usually examined in conjunction with studies on the rate of respiration. Ordin & Jacobson (81) conducted a study of the effects of inhibitors on respiration and the absorption of both the cation and anion of potassium bromide by excised barley roots. Absorption of both ions was inhibited by substances which inhibit glycolysis and the Krebs cycle. With the exception of 95 per cent CO in the light, all agents used inhibited respiration less than ion absorption. For most inhibitors tested, inhibition of subsequent potassium absorption was equal to or greater than that of bromide. Potassium arsenite pretreatment inhibited bromide absorption slightly more than that of potassium. Potassium cyanide pretreatment inhibited bromide absorption more than potassium absorption at low levels of inhibition and was equally inhibitory for both ions at high levels of inhibition. Separate but parallel mechanisms are postulated for the absorption of both potassium and bromide ions.

The light-reversible carbon monoxide inhibition of ion absorption reported by Ordin & Jacobson (81) and earlier workers [Sutter (115); Weeks & Robertson, (118); Robertson, Wilkins, & Weeks (92)] and other evidence linking absorption with the cytochrome system [Lundegårdh (70); Honda (45)] focus attention on the mitochondria, since they contain cytochrome oxidase as well as other respiratory enzymes. Bartley, Davies & Krebs (8) and Bartley & Davies (7) have demonstrated active transport of ions by subcellular particles of animal origin (kidney cortex). Mudge (78) has recently reviewed electrolyte transport in isolated mitochondria of animal origin.

Long & Levitt (68) exposed potato tuber slices to labeled calcium and phosphate for 24 hours and upon subsequent fractionation found marked accumulation of both ions in the mitochondria and microsomes. They suggest that ions may be initially adsorbed on the surface of the particles which subsequently may be carried to the tonoplast surrounding the vacuole, this step being followed by their release into the vacuole. Somewhat similar views had been advanced by Arisz (2). In experiments on the incorporation of P^{32} into the lipid phosphorus fraction of mitochondria isolated from germinated peanut cotyledon homogenates, Mazelis & Stumpf (74) showed that the first step is the esterfication of P^{32} into ATP^{32} (adenosinetriphosphate). This step is oxygen dependent and sensitive to DNP (dinitrophenol). It requires an adenine nucleotide, magnesium ions, and a Krebs cycle intermediate. Subsequent steps in the process of incorporation into lipid phosphorus, and the phospholipid which becomes labeled are unknown. Magnesium is required for these steps, and DNP is without effect.

Robertson, Wilkins, Hope, & Nestel (93) studied respiratory activity and ionic relations of mitochondria isolated from carrot and beet tissues. Both cations (sodium more than potassium) and anions (chloride) were somewhat concentrated at low, but not at higher concentrations of the ions in the external solution. The authors favor the interpretation that cations are held by negative exchange spots of the mitochondria while chloride uptake is by an accumulatory mechanism. In discussing the possible role of mitochrondria in salt accumulation by plants, these authors express views similar to those of Long & Levitt (68). Binding of ions by mitochrondria does not, however, in itself explain secretion into the vacuole, which may be the crucial step in absorption by vacuolated cells if, as the recent findings on "free" or "outer" space suggest, the cytoplasm is quite permeable to ions. Clearly, interpretations of such results concerning mitochondria and their possible bearing on ion transport in cells and tissues must be regarded as speculative, for the time being. However, since respiratory enzymes involved in ion transport are intimately associated with the mitochondria, the study of the ionic relations of mitochondria is sure to be one of the fruitful approaches to the problem in the years ahead. The recent comprehensive review on plant mitochondria by Hackett (37) will be invaluable in providing the background for further studies.

There are numerous and complicated effects of the ionic environment on the activity of mitochondrial enzymes [Laties (63)] which are not necessarily related to ion transport. Ionic effects on the activity of cytochrome oxidase may be no exception. Riley (90) investigated the effect of the ionic environment on the activity of enymes obtained from mouse melanomas. Sodium chloride over a certain range of concentration increased the activity of the cytochrome system, but not that of the succinoxidase or dopa oxidase systems. Butler (19) reported similar findings for a particulate fraction from pea seedlings. Weinstein, Robbins & Wainio (119) have reported that beef heart cytochrome oxidase shows maximal activity in a phosphate buffer of pH 6.0 with a cation concentration of 0.112 M. Activity was related neither to total ionic strength nor the anion concentration of the buffer. For cytochrome oxidase of sunflower leaf tissues, they found a pH optimun of 6.0 and a cation concentration optimum at pH 6.2 of about 0.130 M. Miller & Evans (77) studied the effect of salt concentration on cytochrome oxidase activity of a particulate fraction from the roots of several species of higher plants. Maximal activity of the enzyme was found at a salt concentration of 0.1 N. Monovalent ions were more effective than divalent ones. These findings are similar to those obtained by Inaoka (55) for cytochrome oxidase from ox heart muscle. It may be pertinent in this connection that salt respiration in barley roots, recently studied by Honda (45) and related to the cytochrome system, can be induced by exposing the roots to cations adsorbed on cation exchange resins, i.e., in the absence of absorbable anions [Epstein (29)].

Effects due to cations in the absence of absorbable anions are a special

instance of a very general case, viz., exposure of roots to solutions from which cations are absorbed in excess of anions, as from solutions of potassium sulfate or potassium phosphate. The reverse may also occur; for example, from solutions of calcium bromide, roots will absorb bromide much in excess of calcium. The metabolic responses to unequal cation and anion absorption in barley roots have been reinvestigated by Jacobson & Ordin (57) and Jacobson (56). In the former paper it was shown that the metabolic response to excess cation absorption was largely in the production of malic acid. When anion absorption was in excess of cation absorption, much but not necessarily all of the compensation consisted in a decrease in tissue malate. Jacobson (56) investigated the fixation of $C^{14}O_2$ by excised barley roots as influenced by equal and unequal cation and anion absorption. When cation and anion absorption were about equal (from KBr), the radioactivity fixed was largely in malate, much as when there was no salt present. Under conditions of excess cation absorption (from KH_2PO_4) more C^{14} was fixed, and the reverse held when anion absorption exceeded that of cations (from $CaBr_2$). The greatest fixation of C^{14} in the organic acid fraction (mostly in malate) occurred in the former, and the least, in the latter with the values for experiments with water or KBr being intermediate. In all types, malate was labeled approximately equally in the α and β carboxyl positions. Graf & Aronoff (36) examined the fixation, by onion and soybean roots, of C^{14} from solutions of $NaHC^{14}O_3$. As with barley, malate showed the greatest activity. With increasing concentrations of KCl in the solution, there was an increase in the radioactivity fixed by soybean roots. The finding is probably related to increased synthesis of malate accompanying excess cation (potassium) over anion (chloride) absorption, as discussed by Jacobson (56). Both Jacobson and Graf & Aronoff (36) point out that quantitative evaluations are difficult because of the inconstancy of the ratio of endogenous (nonradioactive) CO_2 production in respiration to fixation of radioactive $C^{14}O_2$. The problem is similar to that encountered in photosynthesizing tissues, which produce O_2 in photosynthesis and simultaneously take up O_2 in respiration; it invites the application of the mass spectrometer technique by which Brown (12) deals with the latter problem. The experiments of Brown & Whittingham (13) show the suitability of the technique for measuring CO_2 exchanges.

The evidence linking ion transport with carriers, on the one hand, and with electron and hydrogen transfers in respiration, on the other, raises the question of how these processes are linked in metabolism. Reference has been made already to Lundegårdh's (70, 71) theory linking respiration with the active transport of anions. Conway (22, 23, 24) has discussed the evidence for a redox pump in the active transport of cations. Originally worked out for yeast, this model seems to have a rather wide application in other systems as well [Conway (24)]. When yeast ferments glucose in an unbuffered medium not containing potassium, succinic acid is excreted. In the presence of KCl, on the other hand, potassium exchanges with the hydrogen

ions previously associated with succinate as succinic acid. Free hydrogen ions appear in the medium, and an equivalent amount of potassium ions is absorbed. The internal pH is raised correspondingly. The process is specific for potassium: a sodium concentration 25 times higher is required to give an equal uptake of sodium. According to the redox pump theory, H atoms from respiratory substrates are transferred to a metallic terminal oxidase which sets hydrogen ions free, retaining the electrons. Potassium ions are transported in a carrier complex, and as the electrons pass inward, potassium ions are set free within the cell.

Lowenhaupt (69) studied calcium transport in the pond weed, *Potamogeton crispus*. This species, like many aquatic seed plants and algae, can utilize externally supplied bicarbonate ions for photosynthesis. Absorption of bicarbonate is linked with calcium absorption and secretion in the leaves. The leaves consist of three layers of cells. It was found that calcium was localized near the external faces of the dorsal and ventral layers, but not at the inner cell walls. Lowenhaupt concluded from a series of experiments that active calcium transport in the light takes place at two membranes. Calcium is absorbed by the plasma membranes at the abaxial leaf surface and excreted by the plasma membranes at the adaxial surface. The steps in active transport at each membrane are as given by Epstein & Hagen (32) and Epstein & Leggett (33), viz., attachment of the ion to the carrier, transfer of the carrier-ion complex across the membrane, and release of the ion at the far side of the membrane. The process is considered to be linked with respiration. The carrier is reduced by a reducing agent, and as a result binds calcium. At the far side of the membrane, an oxidizing agent produced in photosynthesis oxidizes the binding group, and the calcium is set free. The reduced form of the oxidizing agent enters the respiratory chain ending in oxygen. This scheme, in essence, resembles a redox pump as discussed by Conway (22, 23, 24), with modifications appropriate to this particular species especially in regard to bicarbonate assimilation and photosynthesis. In the manner in which they link ion transport with electron and hyrogen transfers in respiration, Conway's redox pump and the anion respiration scheme of Lundegårdh (70, 71) resemble each other. However, the redox pump is not restricted to transport of anions, and it exhibits ion selectivity as an integral part of the mechanism.

Growth.—Steward & Millar (110) have used the technique of tissue culture in investigations of ion absorption, since according to these authors there is an intimate relation between growth and absorption of ions. Secondary phloem of the carrot root can be cultured under sterile conditions, with little growth occurring, or, if supplied with appropriate nutrient substrates and coconut milk factor, it can be made to grow profusely. Contrary to expectation, nongrowing carrot explants, per unit weight, absorbed more cesium than the dividing cells of the growing tissue cultures. This led to the conclusion that two distinct mechanisms are involved in the absorption, depending on the stage of growth of the tissue. During active cell division,

ions are bound by binding entities constantly synthesized in the cytoplasm, and, during the subsequent phase of maturation and vacuolation, ions are secreted into the vacuoles [cf. Arisz (2, 5, 6)]. Another instance of a difference between immature and mature tissue in relation to absorption is found in the gradients in respiration and salt respiration of barley roots, demonstrated by Honda (45). Respiration per segment decreased with increasing distance from the tip, but salt-induced respiration increased. In both these instances, the older, vacuolating cells exhibit the familiar characteristics of accumulation as the process is ordinarily understood. However, Brouwer (11), in experiments with *Vicia faba*, found the salt-induced respiration highest in the apical region, and progressively lower at increasing distances from the tip.

On the basis of the work with growing and nongrowing tissue explants, Steward & Millar (110) have advanced a hypothesis to explain the observed relations between growth and ion absorption. Glutamyl peptides act as ion carriers and are transported to the tonoplast where the amino acid residues and ions are bound to nucleic acid surfaces, glutamine being liberated. While new nucleic acid surfaces are being created, the amino acids and associated ions remain bound and no accumulation in the vacuole takes place. When further synthesis of nucleic acid ceases, the bound amino acids are "peeled off" in protein synthesis, and through formation of α-peptide bonds, the sites which bound the ions cease to exist, and the ions are liberated. However, since these events presumably occur in the cytoplasm external to the tonoplast the question still remains whether the tonoplast, in this scheme, is regarded as the diffusion barrier, and, if so, how the ion is transported across it. Protein synthesis at the inner surface of the tonoplast, facing the vacuole, seems an unlikely possibility. Hanson & Bonner (39) found a reciprocal relation between absorption of salt and of water by Jerusalem artichoke tuber tissue, reminiscent of the findings of Steward & Millar (110) concerning absorption of cesium by static and growing (water absorbing) tissues. Treatment ot the tissue with 2,4-dichlorophenoxy-acetic acid (2,4-D) promoted water uptake but decreased the rate of salt absorption. The authors postulated that the mechanisms leading to water uptake and salt absorption are distinct, but that both depend on a common energy pool. In experiments with *Avena* coleoptile sections, however, Ordin, Applewhite, & Bonner (82) demonstrated that osmotic factors adequately account for auxin-induced water uptake.

TRANSLOCATION THROUGH THE ROOT

The preceding discussion of active ion transport in plant roots has essentially been in terms of cellular physiology. The kinetics of the process were found to be consistent with the view that a reversible binding of the ion by specific sites of carriers is followed by an essentially irreversible breakdown of the ion-carrier complex. Labeled ions absorbed by the active carrier mechanism were found to be largely nonexchangeable with unlabeled ions

of the same species. This being so, the question arises how a cell in the interior of the root can absorb ions from the cells surrounding it, if these cells had previously absorbed the ions irreversibly. Epstein (31) has shown that there is in fact no need for assuming that cells within the root absorb ions which had previously been actively absorbed by other cells. He found that 24 per cent of the total tissue water was accessible to a variety of ions by diffusion, and demonstrated depletion of ions from this "outer" space through absorption by the active mechanism, i.e., their transfer from the "outer" to the "inner" space where they are no longer subject to loss by diffusion or exchange. He concluded that the "inner" spaces of the individual cells, through the active carrier mechanism, absorb ions from a common pool, viz., the solution in the "outer" space of the tissue, this solution in turn being in diffusion equilibrium with the external solution.

According to this view, then, there is no need for assuming a chain of absorption, secretion or "leakage," and absorption by a neighboring cell, in order to account for absorption by all cells of the root. Neither is such a mechanism required for translocation of the ions through the root and on to the shoot, and throughout the various parts of the shoot, according to Hylmö (53). This author's experiments dealt with the effect of transpiration on the uptake of calcium and chloride by pea plants. The shoot was found to be essentially dependent on the transpiration stream for its supplies of calcium and chloride. The effect of transpiration was considered to be direct: the ions are drawn through the root by mass flow in the transpiration stream, without intervention of the active absorbing mechanism of the root. Ions reaching the shoot in this manner are then actively absorbed by the individual cells of the shoot. The ascending sap in the transpiration stream presumably passes through the xylem vessels, and the question remains how the ions enter the vessels. Also, Hylmö's (53) scheme as outlined so far provides no mechanism for the bleeding phenomenon observed with cut stems, and particularly fails to explain the fact that the concentration of the bleeding sap may be higher than that of the medium bathing the roots [Broyer (15)]. This difficulty Hylmö (53) meets by postulating a subsidiary mechanism. As vessel elements mature and their transverse upper walls rupture, the plasma surfaces remain functional, but are now open at the top. These membranes continue to transport ions, as before, but since they are open at the top, the ions are now caught in the transpiration stream.

Like Hylmö (53), Brouwer (11), in experiments with *Vicia faba*, found marked effects of transpiration on the uptake of ions, but his interpretation of the effects is quite different. Brouwer (11) concluded that salt uptake is an entirely active process, at high as well as low suction tension. At increasing suction tensions in the vessels, there occurs an increase in conductivity for water and anions, and the uptake of anions increases in proportion to the increase in water conductivity. Hylmö (54) in turn analyzed Brouwer's (11) data and concluded that they support Hylmö's (53) concept that the

transpiration component of ion uptake is passive and independent of accumulation in the root. Van den Honert, Hooymans, & Volkers (47) concluded from experiments with corn that there was no influence of transpiration on the rate of absorption of ammonium, potassium, nitrate, and phosphate. The difference between these findings and those of Hylmö (53) and Brouwer (11) probably is due to differences in experimental conditions and, to a lesser extent, to the different species used. In particular, van den Honert, Hooymans, & Volkers (47) used lower concentrations than the other two authors. Conceivably active absorption by the roots predominated, so that the transpiration stream was largely depleted of ions, and no increase in total uptake with increased transpiration was possible. As these authors point out, their results do not necessarily conflict with Hylmö's (53, 54) interpretation.

Several other papers indicate a considerable degree of independence of ion translocation through the root from active ion absorption by the root, i.e., by the cells of the root. Wiebe & Kramer (122) found little upward translocation of various labeled ions supplied to the root tips of barley plants, although absorption was rapid in this region. Maximal translocation occurred from a region several centimeters behind the root tip where the xylem is mature and where water flux may be expected to be maximal, also. Hopkins, Specht, & Hendricks (52) concluded that the rate of transfer of ions from the roots to the shoots was independent of aerobic mechanisms in the roots. Broyer (15) pretreated barley plants with nonradioactive bromide, and on subsequently exposing them to labeled bromide found that radioactivity in the sap was less than in the exudate from the decapitated plants. He concluded that the vacuoles, being essentially closed systems and already high in bromide because of the pretreatment, were being by-passed by the labeled bromide supplied later. The conclusions concerning carrier-mediated transport reached by Epstein & Hagen (32) and Esptein (28) from experiments on absorption by roots were criticized by Russell (95) on the basis of short-term experiments on absorption and translocation to the shoot. Since the latter is of a composite nature, there is no justification for Russell's application of his data to results concerned with a single, specified process, viz., absorption by the root cells. It was precisely to avoid ambiguities such as these, which are apt to loom large in short-term experiments, that Hoagland and his collaborators (42) developed the excised root technique of short-term experimentation for dealing with active ion transport at the level of cellular physiology. It seems, in general, that Russell (95) may have been unaware of the evidence for a passive transference of ions through the root.

CONCLUSION

We may consider briefly what happens when roots are immersed in a solution differing in composition from the previous medium. Both cations and anions in the "outer" or "free" space of the tissue diffuse out, and others from the new solution diffuse in, until equilibrium is attained. Cations pre-

viously adsorbed on the exchange surfaces of the root exchange for others from the new solution, until equilibrium is established. At equilibrium, the concentration of diffusible ions in the "outer" space will be essentially equal to their concentration in the external solution if the latter is high. If the concentration of the external solution is low, the distribution of the ions in the "outer" space may be modified by Donnan effects. Diffusible cations in the "outer" space exchange constantly with those constrained by the electro-negative exchange spots. Anions show no appreciable reversible exchange on exchange surfaces. Considerable evidence suggests that in some tissues, at least, the "outer" or "free" space is located in the cytoplasm. In that case, the outer membrane of the cytoplasm must be regarded as quite permeable to those ions that have access to this space.

Ions having reached this space in a passive and reversible manner, by diffusion and exchange, are bound by binding sites of carriers. The carrier sites exhibit considerable selectivity for certain ions or groups of ions. The ion-carrier complexes traverse membranes not permeable to the free ions, and at the far side of the membranes, the ions are released to "inner" spaces in a rate-limiting, essentially irreversible step. These conclusions apply to cations and anions alike. It is customary to identify what has here been called "inner" spaces with the vacuoles, but other entities, such as mitochondria, may also be "inner" spaces in this sense. The evidence for the carriers is entirely indirect. The chemical identity of the carriers and the manner in which their generation and turnover are linked with respiration are unknown. Transference of ions within the root and through the root to the shoot exhibits no simple relation to absorption of ions by the cells of the root.

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ENERGY TRANSFORMATIONS IN PHOTOSYNTHESIS[1,2]

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Introduction

In the last five years the application of physicochemical techniques has made possible great progress towards an understanding of the mechanism of photosynthesis. Present evidence, to be discussed in the following sections, suggests that the reactions of photosynthesis may be divided into four groups. (*a*) The light reactions, leading to the formation of a substance of reducing potential, HX, and one of oxidizing potential, Z.

$$\text{Light} + X + HZ \rightarrow HX + Z$$

(*b*) The dismutation reactions, utilizing a fraction of the HX and Z to generate "energy rich" phosphate bonds in the form of adenosinetriphosphate (ATP) from adenosine-diphosphate (ADP) and inorganic phosphate (P]).

$$HX + Z + n[ADP + P_i] \rightarrow X + HZ + n\text{ATP}$$

(*c*) Carbon dioxide fixing and reducing reactions, utilizing HX and ATP to form a carbohydrate (CH_2O).

$$CO_2 + 4H + m\text{ATP} \rightarrow [CH_2O] + m(ADP+P_i)$$

(*d*) Reactions leading to oxidation of the hydrogen donor: (i) in chlorophyll *a* containing organisms,

$$4Z + 2H_2O \rightarrow 4HZ + O_2$$

(ii) in purple bacteria, which have an oxidizing substance Z′, different from that in algae,

$$4Z' + \text{"substrate"} \rightarrow 4HZ' + \text{"oxidized substrate."}$$

The mechanism of carbon dioxide incorporation and reduction is fairly well established. For this reason we shall discuss first the reactions of group (*c*). Evidence has also been obtained about the mechanism of the dismutation reactions, but still little is known about the mechanism of the light reactions and next to nothing about the dehydrogenation of water. For a survey of the whole field of photosynthesis we refer to Rabinowitch's invaluable monograph (1).

[1] The survey of the literature pertaining to this review was completed in October, 1955.

[2] The following abbreviations will be used: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; DPN, diphosphopyridine nucleotide; DPNH, diphosphopyridine nucleotide (reduced form) FMN, flavin mononucleotide; P_i, inorganic phosphate; PN, pyridine nucleotide; PNH, pyridine nucleotide (reduced form); TPN, triphosphopyridine nucleotide; TPNH, triphosphopyridine nucleotide (reduced form).

Reactions of Carbon-Dioxide in Photosynthesis

Higher plants and algae are able to photosynthesize with a remarkably high rate in air or in water equilibrated with air. In these media, the carbon dioxide concentration is only 0.03 per cent, or 10^{-5} *M*. At this concentration, the photosynthetic rate for many plants, including the green alga *Chlorella*, in strong light is about equal to or more than half the rate attained at "saturating" carbon dioxide concentration [cf. Rabinowitch (1, p. 892)].

Before being reduced by a photosynthetic reductant, carbon dioxide or a carbonic acid form is bound to a cell constituent, the so-called carbon dioxide acceptor. It has been suggested that the binding may take place through a reversal of the decarboxylation of respiration.

$$RH + CO_2 \rightarrow RCOOH$$

However, the equilibrium of this type of reaction, even at one atmosphere pressure of carbon dioxide is in general towards decarboxylation [cf. Rabinowitch (1, p. 184)].

Extensive experiments, mainly by Calvin, Benson, Bassham and coworkers, on incorporation of radioactive carbon in green cells in the light revealed that the photosynthetic carboxylation was different from any previously known carboxylation reaction. Bassham *et al.* (2) gave a comprehensive scheme for the reactions leading to the reduction of carbon dioxide. This scheme was based on the kinetics of C^{14} incorporation in short-time experiments and on carbohydrate conversions catalyzed by plant extracts.

For a discussion of the energy transformations in carbon dioxide fixation, we shall use here a simplified version of this scheme, writing C_n for a carbohydrate with n carbon atoms, and $C_n\textcircled{P}$ and $\textcircled{P}C_n\textcircled{P}$ for carbohydrates, phosphorylated at one and both ends, respectively. $\textcircled{P}$ is $-OPO_3H_2$.

Carbon dioxide reacts with ribulose-diphosphate to give two molecules of 3-phosphoglyceric acid:

$$CO_2 + \textcircled{P}C_5\textcircled{P} \rightarrow 2C_3CO$$

Phosphoglyceric acid is reduced to phosphoglyceraldehyde probably by reduced pyridine nucleotide (PNH) and ATP, which are generated by reactions driven by the light:

$$C_3CO\textcircled{P} + 2HX + ATP \rightarrow C_3\textcircled{P} + H_2O + 2X + ADP + P_i$$

We shall return to these two reactions, since they are the most important energy transformations in the carbon dioxide reactions. The $C_3\textcircled{P}$, phosphoglyceraldehyde, is partly converted to another "carbohydrate" $C'_3\textcircled{P}$, which reacts with phosphoglyceraldehyde to give glucosediphosphate:

$$C_3\textcircled{P} + C'_3\textcircled{P} \rightarrow \textcircled{P}C_6$$

The result of the preceding reactions is that one diphosphorylated C_5 is converted with the absorption of one carbon dioxide molecule into a diphos-

phorylated C_6. After a short time of photosynthesis the supply of $\textcircled{P}C_5\textcircled{P}$ will be exhausted, and it has to be regenerated from the two available carbohydrates, C_3 and C_6. Ignoring for a moment, for the sake of simplicity, the presence of phosphate groups and possible differences between carbohydrates of the same number of carbon atoms, we may write this regeneration as follows:

$$C_3 + C_6 \rightarrow C_4 + C_5$$

C_4 is converted by the following reactions:

$$C_4 + C_6 \rightarrow C_3 + C_7 \rightarrow 2C_5$$

The conversion of the C_4 sugar is that suggested by Vishniac (3) and Horecker & Mehler (4). The conversion suggested by Bassham *et al.* (2),

$$C_4 + C_3 \rightarrow C_7$$

has so far not been observed *in vitro*. Both modifications seem to be in accordance with the experimentally found C^{14} labeling.

The postulated conversion of C_6 and C_3 to C_5 is the reversal of certain reactions of the pentose pathway for glucose oxidation. The reactions of the pentose pathway, which occur in a variety of cells including animal ones have been reviewed by Horecker & Mehler (4). The reversal of the C_5 to C_6 conversion or a similar one occurs in photosynthesis. The oxidative step in the pentose cycle is $C_6 + 2TPN^+ \rightarrow C_5 + CO_2 + 2TPNH + 2H^+$, a reaction quite different from the reversal of the photosynthetic reduction of phosphoglyceric acid. The equilibrium of the reaction of the pentose cycle is far to the side of oxidative decarboxylation and thus is "irreversible." Various enzymes, known to catalyze carbohydrate conversions of the pentose cycle have also been isolated from plant extracts (4).

Thus it seems that the photosynthetic cycle is built up out of: (*a*) an enzyme system for the $C_6 \leftrightarrows C_5$ conversion, similar or identical to that in the pentose cycle; (*b*) the enzyme system of glycolysis which brings about the $C_6 \leftrightarrows C_3$ conversion and the reversible oxidation of C_3; (*c*) enzymes catalyzing reactions involving the carbon dioxide acceptor. Many nonphotosynthetic organisms contain systems (*a*) and (*b*), and it may be asked whether all three systems are present in a nonphotosynthetic organism. An indication that this may be true is provided by Santen & Vishniac's (5) experiments with an extract of the nonphotosynthetic bacterium *Thiobacillus thioparus*. *Thiobacillus* is able to synthesize its organic compounds from carbon dioxide, the energy being provided by oxidation of inorganic sulfur compounds. Extracts of this bacterium were found to catalyze the incorporation of $C^{14}O_2$ into phosphoglyceric acid in the presence of ribulose diphosphate.

Substances required for the reduction of one carbon dioxide molecule.— For every six carbon dioxide molecules absorbed, one C_6 sugar is produced in steady state photosynthesis. The C_6 sugar is formed in the diphosphorylated state. Thus, upon storage two phosphate groups are lost, which are fed back into the cycle by addition to $C_5\textcircled{P}$ via ATP. This reaction requires

two energy-rich phosphate groups in the form of two ATP molecules per six carbon dioxide molecules, or one-third ATP per carbon dioxide molecule. Only one in six diphosphorylated glucose molecules produced by the reduction of phosphoglyceric is stored; the five remaining molecules are converted to ribulose diphosphate. The conversion reactions are known to take place with monophosphorylated sugars.

Vishniac (3) postulated the reaction,

$$\text{Ⓟ}C_6\text{Ⓟ} + C_5\text{Ⓟ} \rightarrow \text{Ⓟ}C_5\text{Ⓟ} + C_6\text{Ⓟ}$$

which not only provides a monophosphorylated C_6 for the sugar conversions, but also takes care of the necessary second phosphorylation of the carbon dioxide acceptor. In this way the minimum requirement of ATP is achieved. So far no enzyme seems to have been found catalyzing the preceding reaction. It is, however, possible that instead of this reaction, two others (2, 4) known to occur in extracts, take place: a hydrolysis removing one Ⓟ from $\text{Ⓟ}C_6\text{Ⓟ}$ and the phosphorylation:

$$\text{ATP} + C_6\text{Ⓟ} \rightarrow \text{ADP} + \text{Ⓟ}C_6\text{Ⓟ}$$

If this is true then one ATP instead of one-third ATP is needed per CO molecule in the sugar conversions, since each turn of the cycle leads to the loss of one Ⓟ and binds one CO_2.

The decrease in free energy of the carboxylation reaction:

$$CH_2\text{Ⓟ}\cdot CO\cdot CHOH\cdot CHOH\cdot CH_2\text{Ⓟ} + CO_2 + H_2O \rightarrow 2CH_2\text{Ⓟ}\cdot CHOH\cdot COOH$$

ribulosediphosphate — 3-phosphoglyceric acid

at pH 7 and for the concentrations of the compounds as they presumably occur in the cells was estimated by Bassham *et al.* (2) at about -11 kcal. per mole. The estimations were based on the free energy of formation from the elements. If we use, instead of Bassham's value for carbon dioxide concentration of 1 per cent, the atmospheric concentration of 0.03 per cent, and assume that the concentrations of the ribulose and phosphoglyceric acid are the same, the free energy change per mole is $1.36 \times \log (1/0.03) = -9$ kcal. Thus, even at 10^{-5} M of carbon dioxide, equilibrium is very much towards the side of carboxylation.

The reduction of phosphoglyceric acid to phosphoglyceraldehyde and the mutual addition of two triose phosphates to form one glucose are presumably the reversal of reactions occurring in glycolysis. In these reactions DPN^+ (or TPN^+) is the oxidizing agent. The direct reduction of the carboxyl group of phosphoglyceric acid by DPNH is energetically not possible. The reduction occurs because additional energy is provided by the hydrolysis of ATP. The reactions are catalysed by the enzyme triose phosphate dehydrogenase, which has been isolated from various sources such as yeast and muscle tissue. First, phosphoglyceric acid is phosphorylated by a reaction with ATP, catalysed by phosphoglyceric phosphokinase.

$$CH_2\text{Ⓟ}\cdot CHOH\cdot COOH + \text{ATP} \rightarrow CH_2\text{Ⓟ}\cdot CHOH\cdot CO\text{Ⓟ} + \text{ADP}$$

This phosphorylation makes a binding energetically possible between 1,3-phosphoglyceric acid and the enzyme phosphoglyceraldehyde dehydrogenase, ESH. This binding takes place to an SH group of the enzyme.

$$CH_2Ⓟ \cdot CHOH \cdot COⓅ + HSE \rightarrow CH_2Ⓟ \cdot CHOH \cdot CO \cdot SE + P_i$$

The "bond" between S— and —C=O is analogous to the "energy rich bond" in acetyl-coenzyme A. DPNH reacts with the compound attached to the enzyme, to give 3-phosphoglyceraldehyde:

$$CH_2Ⓟ \cdot CHOH \cdot CO \cdot SE + DPNH + H^+ \rightarrow CH_2Ⓟ \cdot CHOH \cdot CHO + ESH + DPN^+$$

For a discussion of the reactions we may refer to Racker (6) [cf. also Boyer & Segal (7)].

The "equilibrium constant" of the over-all reaction at pH 7 may be written as follows:

$$K = \frac{(\text{3-phosphoglyceric acid})(\text{ATP})(\text{DPNH})}{(\text{3-phosphoglyceraldehyde})(\text{phosphate})(\text{ADP})(\text{DPN}^+)}$$

K was experimentally determined by Meyerhof & Oesper (8) at pH 7.8 and was found to be about 3×10^3.

If (ATP) = (ADP) and (DPNH) = (DPN^+) and (phosphate) = $(1/3) \times 10^{-3}$ *M*, the equilibrium concentration of phosphoglyceraldehyde will be equal to that of phosphoglyceric acid. At a more acid pH the equilibrium will be towards the side of phosphoglyceric acid and, in order to have a sufficient concentration of phosphoglyceraldehyde, the ATP and DPNH concentration should be greater than that of ADP and DPN^+, while the concentration of inorganic phosphate should be low. Without ATP the reduction of phosphoglyceric acid would take place with a positive free energy of more than 10 kcal. per mole and the resulting extremely low concentration of phosphoglyceraldehyde would slow down very much the rate of conversion of phosphoglyceraldehyde.

The reactions of the cycle leading to the formation of C_5 from C_3 and the storage of C_6 compounds possess probably a standard free energy near zero, so that the cycle will run at a good rate without the necessity of extremely low concentrations of certain intermediates and very high ratios of DPNH/DPN^+ and ATP/ADP.

Progress made in the isolation and identification of enzymes in this cycle was discussed by Vishniac (3). Phosphoglyceraldehyde dehydrogenase and pyridine nucleotides have been demonstrated in leaf extracts. DPN^+ and TPN^+ were found by Anderson & Vennesland (9) to be present in leaves in about equal concentrations. In colorless tissues the concentration of TPN^+ usually is much lower than that of DPN^+.

Arnon *et al.* (10) found that "whole" chloroplasts, prepared by grinding of spinach leaves in isotonic solution, were able to reduce CO_2 in the light. Whatley *et al.* (11), by exposing during a short time whole chloroplasts to distilled water, extracted the following substances: phosphoglyceraldehyde

dehydrogenase, carboxy dismutase, phosphoglycerylkinase, phosphopentokinase, phosphoglucomutase, and phosphorylase. Carbon dioxide reduction stopped after treatment with distilled water but returned upon addition of the extract. Addition of either ATP, TPN, or DPN, or phosphorylated sugars further increased the CO_2 reducing capacity even above that of "whole" chloroplasts. These experiments indicate that carbon dioxide fixation occurs in the chloroplasts and that the phosphoglyceric acid reduction presumably is catalyzed by phosphoglyceraldehyde dehydrogenase. The findings are consistent with the scheme for carbon dioxide fixation discussed above. The stimulation by both DPN^+ and TPN^+ suggests that both coenzymes participate in photosynthesis, although this is not certain because it is possible that one coenzyme is enzymatically converted into the other.

Norris *et al.* (12) studied the distribution of radioactive carbon after 5 and 10 min. photosynthesis with $C^{14}O_2$ in a great number of different chlorophyll containing organisms, among others, higher plants and green, blue, and red algae. The results suggested that the basic pattern of photosynthesis was the same for all these photosynthesizing organisms. In purple bacteria the path of carbon is probably more complicated than in algae. Stoppani *et al.* (13) found that not only phosphoglyceric acid, but also malic acid was early labeled in the photosynthesis of *Rhodopseudomonas capsulatus* in 4 per cent $C^{14}O_2$ and 96 per cent hydrogen [cf. also Glover *et al.* (14)]. The labeling of malic acid reminds one of the reaction catalyzed by Ochoa's "malic enzyme" (15). However, Ochoa's reaction does not seem to proceed at a high rate, and the equilibrium, even at 5 per cent CO_2, is towards decarboxylation [Harari *et al.* (16)]. Tchen & Vennesland (17) studied another reaction, fixing CO_2 in malate with very good efficiency. The over-all reaction, which was catalyzed by wheat germ extract was:

$$\text{phosphoenol pyruvate} + CO_2 + DPNH \rightarrow \text{malate} + DPN^+ + H^+ + P_i$$

The reaction proceeds with oxaloacetate and inosine tri- and diphosphates as intermediates. Apparently the free energy of the hydrolysis of the "energy rich" phosphate bond favors carboxylation. Van Niel has reviewed (18) previous experiments with purple bacteria which also indicated that the phosphoglyceric acid cycle is not the only cycle incorporating $C^{14}O_2$ into bacteria. Unlike green plants, purple bacteria do not primarily depend upon the atmosphere with its low CO_2 pressure as a carbon source. The carbon dioxide pressure in their environment may be appreciable, since often other fermenting organisms are present. Thus a carbon dioxide fixing system, requiring for good efficiency a carbon dioxide pressure higher than that of air might play a role in bacterial photosynthesis.

The reducing reactions observed so far in the photosynthesizing organisms studied, appear all to require the same agents, reduced pyridine nucleotide (PNH) and ATP. We are thus faced with the question how these compounds are produced in photosynthesizing cells.

Photochemical Reactions of Chlorophyll in Solution

If chlorophyll *a* participates in the photochemical reactions of photosynthesis, then it is called upon to produce the oxidizing and reducing substances Z and HX (see introduction) which yield PNH, ATP, and oxygen.

Illuminated chlorophyll has been found to be capable of sensitizing a great variety of oxidation-reduction reactions. For an extensive discussion and for references, especially concerning the numerous Russian papers, only a small fraction of which were available to us in translation, we may refer to Rabinowitch (1, Vol. II, 2) and also to the review by Lumry *et al.* (19).

Krasnovsky (20) found that illumination of chlorophyll in pyridine in the presence of ascorbic acid caused the disappearance of the red absorption maximum. Upon darkening, the original absorption was slowly restored. The pink product formed upon illumination of chlorophyll *a* has an absorption band at 520 mμ and a minor one at 585 mμ [Evstigneev & Gavrilova (21)]. In chlorophyll *b* the red maximum disappeared, too, upon illumination, and a new band arose at 565 mμ. It was also found that at least part of the absorption in the red maximum of chlorophyll *a* could be restored in the dark by addition of oxidized riboflavin or DPN^+. Evidence was given that in the light, DPN^+ was reduced with the concomitant oxidation of ascorbic acid. In this reaction an increase in free energy of 6.5 kcal. occurs per equivalent, as compared to 26 kcal., when water is oxidized to oxygen with the concomitant reduction of DPN^+. The experiments indicate that illuminated chlorophyll oxidizes ascorbic acid and that subsequently the reduced chlorophyll reduces riboflavin or DPN^+ in a dark reaction. The reduction of methyl red by phenylhydrazine in illuminated chlorophyll solutions may proceed by an analogous mechanism [cf. Watson (22); Livingston & Pariser (23)].

Rabinowitch & Weiss (24) observed a "bleaching" of chlorophyll in methanol after addition of ferric chloride. The original absorption spectrum is restored by addition of ferrous chloride. Rabinowitch (1, p. 465) has given reasons, making it plausible, that the disappearance of the red band is caused by an oxidation of nucleus IV of chlorophyll *a*. The oxidation is reversed by ferrous chloride. Holt & Jacobs (25) confirmed Schneider's (26) observation that bacteriochlorophyll can be easily oxidized. They used ferric chloride to oxidize a bacteriochlorophyll solution in methanol and isolated by chromatography a predominant green pigment. The absorption spectrum in ether showed maxima at 434 and 674 mμ, reminding one of the absorption spectrum of chlorophyll *a*. The bacteriochlorophyll maxima were at 770, 392, and 357 mμ. The oxidation may have taken place in nucleus II, which changes the conjugated bond system into that of chlorophyll.

Rabinowitch (1, p. 488) observed about a 1 per cent bleaching of the red chlorophyll maximum in methanol in the presence of a mixture of ferric and ferrous chloride. Porret & Rabinowitch (27) observed a light induced bleaching of 1 per cent in methanol also without adding reductants (or

oxidants). The latter type of bleaching was, in contradistinction to that in the presence of ferric plus ferrous chloride, completely inhibited by a small trace of oxygen. Livingston and co-workers extensively studied the kinetics of this type of bleaching. Very recently Livingston (28) was able to photograph with a weak flash (cf. 29) the absorption spectrum of an oxygen-free chlorophyll solution within a msec. after illumination of the solution with a medium intensity flash. Very pronounced changes occurred: a decrease in blue maximum and an increase around 460 mμ for chlorophyll *a* and around 490 mμ for chlorophyll *b*. Because, in general, there is no correlation between fluorescence of chlorophyll and reactions sensitized by it, it seems likely that not the fluorescent state but some lower energy rich state or states of chlorophyll participate in the chemical part of the discussed reactions of chlorophyll *in vitro*.

It has been suggested that this state is a triplet state. Triplet states have much longer natural life times than singlet states, such as the fluorescent one; thus the molecule in the triplet state has a better chance to participate in collision reactions. The same is true for a molecule in a state in which the energy is stored in the form of potential energy of atomic nuclei. There is experimental evidence suggesting the occurrence of a triplet state of chlorophyll *b* in the infrared region. Calvin & Dorough (30) and Becker & Kasha (31) reported delayed infrared emission of chlorophyll *b* in a glassy solvent at −180°C. The maximum occurred at 865 mμ [Becker & Kasha (31)]. According to these authors, the light emission is attributable to slow fluorescence emitted in the electronic transition from the lowest triplet to the lowest singlet state. No slow emission was found for chlorophyll *a*, but it is possible that a triplet state exists and is responsible for some of the reactions of chlorophyll *a in vitro*.

In resumé, we may say that experiments with chlorophyll *a in vitro* show that illuminated chlorophyll can catalyse reversible oxidation-reduction reactions. Not the fluorescent state, but a lower state, participates directly in the chemical part.

The reactions of chlorophyll in solution cannot easily be related to the reactions in photosynthesis. Even in Krasnovsky's reaction the potential bridged is four times smaller than that in photosynthesis and also the efficiency per quantum absorbed is rather low. It will be necessary to imitate more closely the conditions in the plant and to provide the right substances to react with chlorophyll. One may wonder whether the study of chlorophyll in solution or of the chloroplast system will open the road to an understanding of the mechanism of the primary reaction of photosynthesis.

Role of the Pigments in Photosynthesis

Energy transfer between different pigments in photosynthesizing cells.—It has long been known that, besides chlorophyll, other pigments were photosynthetically active, but it was a matter of conjecture whether these pigments were active through transferring their energy to chlorophyll or through direct photochemical participation. As recently as 1950, Haxo &

Blinks (32), who had found that chlorophyll *a* in red algae was less active in photosynthesis than phycoerythrin, stated, "In view of the poor utilization of light absorbed directly by chlorophyll, the energy transfer from phycoerythrin to chlorophyll seems unlikely, since the latter process would have to be more efficient than the former." However, spectral fluorescence measurements showed that the energy was transferred to chlorophyll *a*, not only in red algae [French & Young (33); Duysens (34, 35)] but also in blue-green algae (35), in the green alga *Chlorella* (35), and in diatoms [Dutton *et al.* (36); Wassink & Kersten (37); Duysens (35); French (38)]. In the various species of purple bacteria investigated (35) the energy was transferred to the bacteriochlorophyll with absorption maximum at the longest wavelength, B 890.

The experiments with red and blue algae could satisfactorily be explained by the additional hypothesis that part of the chlorophyll *a* was inactive in photosynthesis, nonfluorescent, and did not receive energy from the phycobilins (35). Experiments based on these species alone are not completely convincing. However, it was pointed out (cf. 35) that the proportionality between the action spectra for photosynthesis and for excitation of chlorophyll *a* or bacteriochlorophyll B 890 fluorescence, observed in all pigment systems studied (35), argues very strongly in favor of the hypothesis that a pigment different from chlorophyll *a* or B 890 becomes only active in photosynthesis through transferring its excitation energy to these chlorophyllous pigments. As an example we may discuss experiments on spectral photosynthesis and fluorescence of the purple bacterium *Rhodospirillum rubrum*, strains 1 and 4. *Rhodospirillum rubrum* was the only species studied, except the species of blue and red algae, for which the interpretation of experiments on energy transfer provided difficulties. Manten & Milatz (39, 40) measured the action spectrum of phototaxis of strain 4 and proposed the hypothesis that this spectrum is proportional to that of photosynthesis. This proposal was supported by the action spectrum of photosynthesis, determined by Thomas (41). The action spectra of phototaxis and photosynthesis showed that light absorbed by carotenoids was active in photosynthesis (and phototaxis), but less so than light absorbed by bacteriochlorophyll. Surprisingly, the seemingly most abundant carotenoid, spirilloxanthin, proved to be inactive, and, instead, the carotenoid 530 (with longest wavelength hump at 530 mμ) was the one active in phototaxis and photosynthesis. To complicate matters, Clayton (42) found spirilloxanthin to be active in phototaxis, but his strain (strain 1) and method of measurement were different from those of Manten & Milatz. Action spectra for excitation of bacteriochlorophyll fluorescence, strain 4, showed great variability (35, p. 38). It appeared that the carotenoid 530 was more active in energy transfer for "young cultures," spirilloxanthin for "old cultures." Measurements of absorption spectra also showed a predominance of carotenoid 530 in young and of spirilloxanthin in old cultures; both carotenoids transferred about 40 per cent of the absorbed quanta to bacteriochlorophyll. It was suggested (35, p. 40) that Manten and Thomas' experiments showed only the activity of the young bacteria con-

taining predominantly carotenoid 530. Van Niel (18) recently reported experiments by van Niel, Blinks & Airth which confirmed this suggestion and explained also Clayton's results satisfactorily. Both strains of *Rhodospirillum* possessed predominantly carotenoid 530 when young, and spirilloxanthin when old. Furthermore spirilloxanthin was found to be active in photosynthesis of those cultures of bacteria in which it was the predominant carotenoid. Thus, in strains 1 and 4, both spirilloxanthin and carotenoid 530 are partly active in photosynthesis through transfer of energy to bacteriochlorophyll, and the apparent discrepancies between previous experiments were caused by a change in the pigment system during growth.

For a general review of the experiments on energy transfer we may again refer to the review by Lumry *et al.* (19) and to the extensive discussion by Rabinowitch (1, vol. II, 2). Also Blinks (43) reviewed certain aspects of the problem.

The transfer of electronic excitation energy probably takes place through the mechanism of induced resonance. If the energy receiving molecule is in the oscillating electric field of the excited energy-transferring molecule, electronic vibration will be induced in the receiving molecule, provided resonance occurs between the oscillating field and the electronic transitions of the receiving molecule. Somewhat differently expressed: the probability of the transfer process is the more efficient, (*a*) the better the overlapping of the fluorescence spectrum of the transferring molecule with the absorption band of the receiving molecule, (*b*) the smaller the distance between the two molecules, and (*c*) the greater the fluorescence yield of the transferring molecule and the specific absorption of the receiving molecule. Förster (44) has given a quantum mechanical theory of the transfer mechanism with the aid of which the transfer efficiency can be calculated. Duysens (35, p. 80) applied this theory to the experimental data and found a reasonable agreement between observed and calculated efficiency of transfer.

As discussed above, energy not transferred to chlorophyll *a* or bacteriochlorophyll 890 in bacteria, is lost for photosynthesis. In these chlorophyllous pigments, as in all other similar pigments [see Pringsheim (45)] the excitation energy is converted into that of the fluorescent state. Thus light energy is probably passed to photosynthesis via the lowest singlet (fluorescent) state of a chlorophyll *a* or bacteriochlorophyll B 890 molecule. Induced resonance causes a rapid transfer of the energy between chlorophyll *a* molecules. Estimates (35) based upon the theory of induced resonance by Förster (44) indicated that the excitation energy in the fluorescent state of chlorophyll *a* is transferred from one chlorophyll *a* molecule to another roughly two hundred times.

The Primary Reaction of Photosynthesis

Now we leave the field of rather well known reactions and mechanisms to enter a frontier area where we have to find our way partly by guessing what is ahead.

The first reaction converting the excitation energy of chlorophyll into chemical energy is probably the production of a reducing and oxidizing compound. It may be asked, whether (*a*) chlorophyll *a* itself participates in a chemical reaction or (*b*) excited chlorophyll transfers its excitation energy by the mechanism of induced resonance to a pigment different from chlorophyll which then participates in a chemical reaction. Small reversible changes in the infrared absorption spectrum of purple bacteria upon illumination (35, 35a) indicate that bacteriochlorophyll B 890 or a fraction of B 890 is changed upon illumination. This suggests that B 890 itself participates in the chemical reactions.

We shall now also make the generally accepted, but not yet proven, assumption that chlorophyll *a* and B 890 directly participate in the chemical reaction. With slight modifications most of the following reasoning remains applicable, if another pigment, to which chlorophyll transfers its energy, reacts chemically. The first chemical reaction may take place (*a*) upon collision of intermediates with energy-rich chlorophyll or (*b*) through a reaction in a stable complex of energy-rich chlorophyll and intermediates. It is unlikely that chlorophyll reacts in its fluorescent state in a collision process, since the life time of this state is extremely short. A relatively long-lived energy-rich state of chlorophyll, such as the triplet state, would have a better chance of reacting by collision during its life time. However, in a following section it will be argued that too much loss of energy occurs in the transition of chlorophyll from fluorescent state to triplet state, to be consistent with the measured quantum requirements. It seems most likely that the primary reaction takes place in a rather stable complex consisting of chlorophyll *a* and intermediates. Such a mechanism was used by Rabinowitch (1, p. 1024 ff.) to discuss the shape of the light curves of photosynthesis and fluorescence. Using Rabinowitch's notation, the reaction is written as:

$$X \cdot Chl \cdot HZ + h\nu \rightarrow HX \cdot Chl \cdot Z$$

where HX and Z are reducing and oxidizing substances. The reaction may proceed in two steps. Excited chlorophyll reacts first, e.g., with X, to give HX and dehydrogenated chlorophyll. Then dehydrogenated chlorophyll reacts with HZ to give chlorophyll and Z. There is some evidence that the changes in the bacteriochlorophyll absorption upon illumination of bacteria are caused by oxidation of part of bacteriochlorophyll (35a, 46). This is consistent with the two step mechanism discussed. Mechanisms specifying X and Z have been given. That proposed by van Niel (cf. 47) has served as a useful guide in many speculations concerning photosynthesis:

$$HOH + E' + E'' + h\nu \rightarrow E'H + E''OH$$

E′ and E″ are acceptors to which H and OH are strongly bound. The primary reaction is followed by several dark reactions: in algae one of these is the dismutation of 4 E″OH into water and oxygen.

The scheme formulated in the introduction is a generalisation and

modification of van Niel's scheme, to which a dismutation reaction had to be added, because of new experimental evidence. It has been suggested (cf. 48) that the primary reaction is the splitting of the S—S bond in thioctic acid, but new evidence makes this unlikely (see section on free energy requirement).

Flashing light experiments.—Determination of the average rates of photosynthesis, measured in illumination consisting of periodic light flashes, makes possible a partial analysis of photosynthetic reactions. For an extensive discussion we may refer to Lumry *et al.* (19). Two types of experiments may be distinguished. In one type, first carried out by Emerson & Arnold (49) for *Chlorella*, the flashes were only 10^{-5} sec. or shorter in duration. At room temperature and nonlimiting CO_2 concentration, maximum oxygen production per flash is attained with dark times between the flashes of a few hundredths of a second. Saturation is just obtained if, on the average, one quantum per approximately 200 chlorophyll molecules is absorbed per flash. In the second type of experiment flashes of 10^{-3} to 10^{-2} sec. were used. The most extensive experiments of this type were done, also with *Chlorella*, by Tamiya & Chiba (50). Surprisingly the dark time required at high flash intensities was of the order of 0.1 sec. Furthermore, the maximum yield per flash at saturating flash intensity appeared to depend upon the temperature, in contradistinction to the maximum yield in corresponding experiments with 10^{-5} sec. flashes.

The results of the experiments with the flashes of 10^{-5} sec. suggest the following mechanism. The reaction by a saturating flash uses up, within 10^{-5} sec., the whole amount of a certain intermediate, which then has to react with other substances to be ready again for reacting in a subsequent flash. The restoring reaction is longer than 10^{-5} sec. This mechanism explains flash saturation and the independence of the yield per flash from the temperature. If the reaction is a one quantum reaction, the amount of intermediate is about 1/200 of that of chlorophyll. If it is assumed that the restoring reaction would take 10^{-2} sec., this would explain the dark time required with 10^{-5} sec. flashes, but it would not be possible to understand why flashes of 10^{-3} sec. duration have a greater yield than those of 10^{-5} sec. Thus we are forced to assume that the restoring reaction is shorter than 10^{-3} sec., say 10^{-4} sec. Then in 10^{-3} sec. the intermediate can react several times, and the yield will be greater than that of a 10^{-5} sec. flash, however intense. A second dark reaction following the restoring reaction has to be postulated to explain the dark times of 0.01 to 0.1 sec. The first reaction takes place within less than 10^{-4} sec., since the sum of the time required for it and the restoration time is of the order of 10^{-4} sec. This short time suggests that it is the primary reaction of photosynthesis. If this is true, then only a small fraction of the chlorophyll (1/200 or less of the total amount) can be present in the form of complexes $X \cdot Chl \cdot HZ$. Energy absorbed by chlorophyll molecules not present in the complexes then is transferred by induced resonance to chlorophyll molecules in the complexes. An efficient transfer is quite possible, especially if the complexes are nonfluorescent (35). A nonfluorescent complex will

trap the excitation energy, since further transfer by induced resonance does not occur, because fluorescence is essential for transfer. If the restoring reaction is:

$$4HX \cdot Chl \cdot Z + 2PN^+ + 2H_2O \rightarrow 4X \cdot Chl \cdot HZ + 2PNH + 4OH + 2H^+$$

then the second reaction, requiring the dark time of 0.01 to 0.1, is the "slowest" of the reactions converting 4OH (or $2PNH+2H^+$) back to $2H_2O+O_2$ (or $2PN^+$). The dark time depends upon the amount of OH or PNH formed per flash.

Hypotheses resembling that formulated above have been proposed before in the literature. Very recently, Franck (51) assumed a similar hypothesis for discussing experiments with flashing light.

Damaschke & Rothbühr (52) used flashes of 10^{-5} sec. of intensities about four times higher than required to attain flash saturation. At these flash intensities also the yield per flash was independent of temperature. They found azide to decrease the yield per flash but not the dark time, which suggests that the complexes themselves were inhibited by azide. Formaldehyde and iodoacetate did not inhibit the complexes, but probably inhibited a reaction subsequent to the restoring reactions.

Kok (52a) who recently carried out precise flash experiments with saturating flashes of a life time down to 3×10^{-4} sec., states that the results of his experiments with many (although not all) cultures of *Chlorella* are quantitatively consistent with the hypothesis of two subsequent reactions, similar to those discussed above.

Change in redox state of chloroplast components upon illumination.—The primary photosynthetic reaction will cause a shift in the oxidation-reduction state of intermediates of photosynthesis. Inversely, these shifts may give information about the primary reaction. The compound of the most reducing potential known to be produced in the light is hydrogen; that with the most oxidizing potential is oxygen. Hydrogen production was observed in purple bacteria [Gest *et al.* (53)] and, after periods of anaerobiosis, in *Scenedesmus* [Gaffron (54)], *Chlamydomonas* [Frenkel & Lewin (55)], and *Chlorella* [Spruit (56)]. Several couples with a normal potential between oxygen and hydrogen appear in the middle column of Figure 1. The more oxidizing couples occur lower in the figure. The left scale indicates the free energy of "combustion" by atmospheric oxygen: one equivalent of the reduced component of the corresponding couple is oxidized to give the oxidized component. The free energy is given for the couple in the normal state at pH 7. Riboflavin, vitamin K (menadione), and ascorbic acid have been demonstrated to stimulate photosynthetic phosphorylation [cf. Arnon *et al.* (57)]. Cytochrome-*f*, or a pigment with a similar absorption spectrum, present in the algae, is reversibly oxidized upon illumination of intact cells [cf. Duysens (58)]. Evidence for reduction of pyridine nucleotide was given in the section on carbon dioxide reactions. The simplest explanation of these observations, but not the only one, is that the primary reductant HX has a potential equal

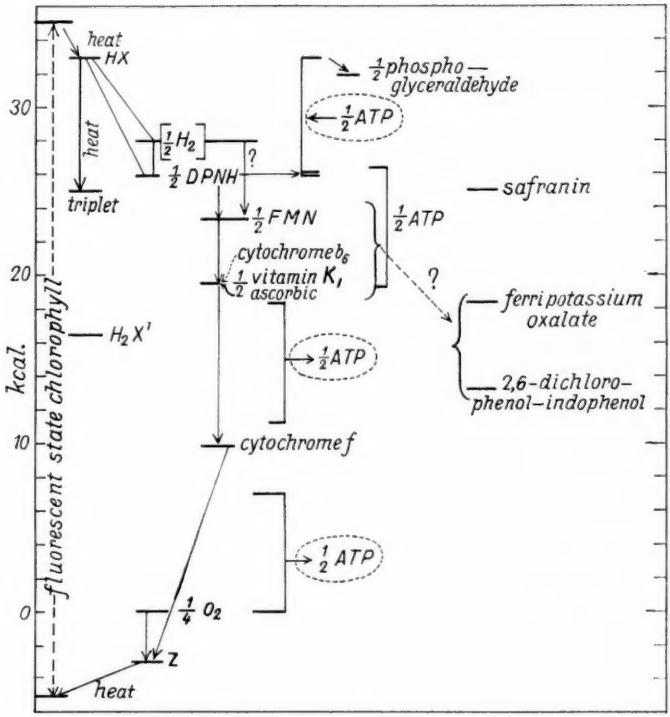

FIG. 1. Scheme showing hypothetical energy transformations in photosynthesis in connection with free energies of "combustion" of reduced intermediates.

to that or more reducing than that of PNH/PN^+, and the primary oxidant HZ/Z has a more oxidizing potential than that of O_2/H_2O. Part of the HX and Z is short-circuited via cytochrome-*f* and via some of the other couples of intermediate oxidation reduction potential. This dismutation reaction yields ATP from ADP plus inorganic phosphate. The main part of the HX reduces PN^+; the corresponding Z dehydrogenates water to give oxygen. The PNH and ATP are used for reduction of phosphoglyceric acid according to the reaction described in the section on carbon dioxide reactions. It is well established that added PN^+ is reduced by illuminated chloroplasts [see review by Vishniac (3)]. This reduction is difficult to observe directly, presumably because of competing intracellular oxidants which oxidize HX or PNH, thus keeping the concentration of PNH low [cf. (58)]. Hill oxidants, some of which are written in the right hand column of Figure 1, are only reduced by illuminated chloroplasts, if their normal potential is less reducing than 0.0

V. [cf. Macdowall (59); Wessels (60)]. This observation may be explained by assuming that these reductants react most rapidly with one of the reduced cellular components of a couple with an oxidation-reduction potential of about zero, such as cytochrome-b_6 (see Figure 1). The concentration or the reactivity of the reduced components of the more reducing couples may be not high enough to cause a measurable rate of reduction of oxidants like safranin, that have a potential more reducing than 0.0 V.

A different primary reaction has been proposed by Wessels (61). He suggested that one quantum produces a reduced vitamin K molecule and a half oxygen molecule. The PNH then is formed by a reaction of ATP with reduced vitamin K. The ATP is produced by a dismutation of part of the reduced vitamin K with part of Z or oxygen. In Wessels' scheme, the observed stimulation of photosynthetic phosphorylation (57) by FMN is difficult to understand since FMN is more reducing than vitamin K. It is possible, however, that the normal potential of FMN has become more positive by complexing with ions of heavy metals [see report by Mahler (62)]. Another more serious difficulty to Wessels' proposal will be discussed in the next section. Strehler (63) earlier suggested a similar type of reaction for building up reduced PNH with the aid of ATP, but he did not specify the primary reductant. His proposals were based on the time course of ATP concentration in intact cells under various conditions. Perhaps his most remarkable observation was that the time course after illumination was the same in the presence and absence of CO_2. Strehler concluded that ATP was produced and utilized essentially independently of carbon dioxide concentration. However, the recent work in the laboratories of Calvin and of Arnon indicates that the rate of utilisation of ATP must depend on carbon dioxide concentration. The absence of an effect of carbon dioxide on the level of ATP indicates that the production of ATP is increased by carbon dioxide by approximately the same amount as its utilisation is decreased. One might suppose that the primary reductant HX has a more reducing potential than PNH, and that upon reduction of PN^+ with two HX, one ATP is produced, which is subsequently utilized in the reduction of phosphoglyceric acid. Figure 1 indicates that this is possible energetically. If this is true, then the reduction of phosphoglyceric acid does not cause any change in the ATP level, but the utilization of ATP in the phosphorylation of carbohydrate must be postulated to be negligible. A different explanation is suggested by Schlegel's (64) experiments. This author found that the ATP level increased in intact cells when oxygen was admitted in the dark after a period of anaerobiosis, but only in the presence of carbon dioxide; the level remained constant when carbon dioxide was absent. This was found for *Chlorella* and *Rhodospirillum rubrum* and also for the nonphotosynthetic bacterium *Hydrogenomonas*. CO_2 may be needed as activator in the production of ATP. A similar activation may be necessary for the generation of ATP in the light. Apparently more evidence is needed before a satisfactory interpretation of Strehler's experiments can be given.

In purple bacteria the primary reductant, HX, may well be the same as in algae or higher plants. It is, however, well established that purple bacteria do not produce oxygen. The most oxidized compound observed may be a cytochrome. With sensitive absorption spectrophotometry, a reversible shift towards oxidation of a cytochrome with a Soret band at 428 mμ was observed upon illumination of *Rhodospirillum rubrum* [Duysens (65)]. This indicated that "cytochrome 428" was an intermediate in the photosynthesis of *Rhodospirillum rubrum.* It was suggested that the cytochrome may participate in the light-induced dehydrogenation of the substrate. Frenkel's (66) finding of photosynthetic phosphorylation in extracts of these bacteria suggests an alternative interpretation: that the cytochrome may be an intermediate in photosynthetic phosphorylation. Chance & Smith (67) confirmed Duysens' observation of the oxidation of cytochrome 428 by light but believed that their difference spectrum in the green region, which was obtained under somewhat different conditions, indicated also the participation of another cytochrome, namely cytochrome-"c_2" [Vernon & Kamen (68)]. Carbon monoxide was found to combine with a cytochrome having a peak at about 428. If this cytochrome is identical with that oxidized in the light, as Chance and Smith believed, then cytochrome 428 may be oxidized either by light in photosynthesis or by oxygen in respiration. The role of the cytochromes extracted from purple bacteria by Vernon & Kamen (68) is not yet clear. Cytochrome-"c_2" has a normal oxidation-reduction potential at pH 7 of +0.3 v. (68), slightly less oxidizing than cytochrome-*f*. It thus seems that the primary oxidant of bacterial photosynthesis may have a higher oxidizing potential than +0.3 v.

Quantum requirement.—The experimentally observed low quantum requirement of photosynthesis restricts the number of possible reaction mechanisms. The quantum requirement may be defined as the smallest number of light quanta necessary per oxygen molecule produced during a fairly long time in light intensities well above those needed for compensation of respiration. The limitations in this definition are imposed because (*a*) in short time experiments, induction phenomena may make uncertain the calculation of a meaningful quantum requirement [cf. Gaffron & Rosenberg (69); Kok & Spruit (70)] and (*b*) in photosynthesis below compensation of respiration, the correction for respiration is relatively great and introduces uncertainties in the calculation and interpretation of efficiency calculations [cf. Kok (71); Bracket *et al.* (72); Bassham *et al.* (73)].

The values of recently determined quantum requirements are about: 4, [Warburg *et al.* (74)]; 5 to 6, [Burk *et al.* (74a)]; 6.1 ± 0.6, [Bracket *et al.* (72)]; 7.4, [Bassham *et al.* (73)]; 12, [Yocum & Blinks (75)]; different values from 6 to 10 [Lung Yuan *et al.* (76)]. Yocum & Blinks' requirement is for certain species of thalloid algae, the other requirements are for *Chlorella.* Warburg's and Burk's values were calculated without a correction for respiration. Of the values of Lung Yuan *et al.*, probably the lowest ones are significant because of rather small variation in the values for experiments with

one culture; furthermore at the wavelengths of measurement, 436 and 541 mμ, the quantum requirement is according to Emerson & Lewis (77) about ten per cent higher than in the red region. A critical discussion of the determination of quantum requirements for *Chlorella* might be possible, if in all investigations the experimental conditions and results were as completely documented and checks were carried out as extensively as in those of Bracket and co-workers. In spite of discrepancies between the results of several laboratories, the recent determinations of the quantum requirement of *Chlorella* [see also Burk *et al.* (74a)] seem to indicate that it is in the range 4 to 8.

Free energy requirement of photosynthetic reactions.—It is now possible, because of accumulated evidence, to give a more specific discussion of energy requirements than could be done a few years ago. The following is based, in part, on discussions by Strehler (63), Franck (79), and Lumry *et al.* (19).

Discussion in the section on energy transfer between pigments indicated that for algae only the energy of the fluorescent state of chlorophyll *a* is available. This may be estimated from the fluorescence spectrum of *Chlorella* (35) to be 38–40 kcal. In the primary process some energy must be lost as heat, which is estimated by Franck (79) at not less than 4 kcal. Thus the increase in free energy of the primary reaction.

$$X + HZ + h\nu \rightarrow HX + Z$$

is probably smaller than $40 - 4 = 36$ kcal.

The mechanism of the dehydrogenation of water:

$$4Z + H_2O \rightarrow 4HZ + O_2$$

is not known, but because of its complexity and the readiness with which this reaction occurs even at a pressure of one atm. oxygen, the free energy loss may well be more than 10 kcal. per mole O_2. If, per mole Z, 3 kcal. are lost, then the free energy decrease of the combustion of HX to X is less than $36 - 3 = 33$ kcal. The free energy decrease of combustion of hydrogen at 1 atm. is 28 kcal. per equivalent, and, according to Burton & Wilson (80), that of pyridine nucleotide is 25.9 kcal. per equivalent (which is half a mole of DPNH). Thus two HX may well be able to reduce one PN^+ molecule. There is a decrease of 14 kcal. or less per mole PNH produced in this reduction, which might be used for the production of one ATP from ADP and inorganic phosphate; the ATP formation may require 13–16 kcal. *in vivo* [cf. Burton & Krebs (81)]. However, it is likely that the free energy decrease in the primary reaction is more than 4 kcal. Unless the primary and subsequent reactions are unusually rapid, a small decrease in free energy will cause a reversal of the primary reaction and a loss of energy through internal conversion or fluorescence of the excited chlorophyll. If the primary reductant has a potential only slightly more reducing than PNH, then, as suggested by the scheme, the reaction between Z and HX may produce 1.5 ATP. Two PNH and presumably three ATP are needed for the reduction of one carbon diox-

ide molecule. This requires six HX and six Z, or six quanta. The calculated quantum requirement would only be slightly more than four, if ATP was generated also between HX and PN^+. Barltrop *et al.* (82) suggested that the primary reaction is the split of the S—S bond in thioctic acid, followed by addition of the H and OH of a water molecule to the two radicals formed. It was thought that the strain in the ring system, of which the disulphide is a part, would lower the bond strength by about 27 kcal., thus making the split by one light quantum possible. However, Sunner (83) recently showed by caloric experiments that the strain is probably only 3.5 kcal. per mole. This fact makes unlikely the splitting of the S—S bond by one quantum. In a recent review of work in Calvin's laboratory (84) thioctic acid only plays the role of reducing agent for PN^+, but the evidence for this too, is as yet uncertain (see section on Methods).

If not the fluorescence state, but the lowest triplet state participates in the primary reaction of photosynthesis, additional energy will be lost. The triplet state of chlorophyll *a* is unknown, but if it is assumed to be at the same distance below the fluorescence state as the triplet of chlorophyll *b*, [Becker & Kasha (85)], then the energy loss is about 8 kcal. The energy available for HX is then 25 kcal., which is not enough to reduce the pyridine nucleotide present to an appreciable extent. HX is only able to reduce another less reducing couple, e.g., cytochrome-b_6. Two reduced cytochrome-b_6 may subsequently reduce one PN^+ with the aid of one ATP. The reduction of one PN^+ then requires $2+2=4$ quanta. Two PNH and three ATP are needed for the reduction of one CO_2. In total, $2\times4+3=11$ quanta would be required in this mechanism to reduce one CO_2 molecule. This number is in contradiction with the experimental evidence.

The quantum requirement would be $4+7/1.5=8.7$, if between cytochrome-*f* and Z, not one half, as suggested by the scheme, but one molecule of ATP were generated per oxidized cytochrome molecule. This seems just possible energetically, albeit not very likely. Since the experimental quantum requirement is probably less than eight it seems unlikely that chlorophyll reacts in the triplet state in photosynthesis. One of the assumptions in the above considerations was that ATP is the smallest coin in the energy economy of photosynthetic organisms. This hypothesis is consistent with present evidence. Of course, a gain of a few kcal. in free energy may be obtained by increasing or decreasing the ratio of the concentrations of the components of an oxidation-reduction couple. If, however, an appreciable gain is desired, the required low concentration of one of the components in general will limit its reaction rate, and thus the rate of the over-all reaction.

A different type of primary reaction is that in which two, instead of one, hydrogens (or electrons) are shifted by one quantum:

$$h\nu + X' + H_2Z' \rightarrow H_2X' + Z'$$

Michaelis and co-workers (cf. 86) demonstrated for a few reactions that apparent two-electron transfers occur by two, one-electron (or one hydrogen) transfers, and it was implied that this might be the usual type of oxidation-

reduction reaction. However, Westheimer (87) recently has given examples of reactions in which two electrons are transferred within a time too short to allow any reaction with a possible intermediate radical and pointed out that for all practical purposes such a reaction is a two-electron reaction. It is possible that many chemical reactions are of this type. If the primary reaction is such a two-electron (or two-hydrogen) transfer, then the reducing compound H_2X' has a free energy of combustion of less than 33 kcal. per mole. Thus the primary reaction cannot be the straightforward reduction of PN^+, which requires 51.8 kcal., or the reduction of vitamin K (61) which requires 39 kcal.

Methods and Miscellaneous Observations

Recently published techniques and observations of possible importance for studies bearing on the mechanism of photosynthesis are given in this section.

Measurement of gas exchange.—A recording apparatus detecting the paramagnetism of oxygen was used for the determination of the quantum requirement of photosynthesis [Bassham *et al.* (73); Lung Yuan *et al.* (76)]. Arnold *et al.* (88) constructed an apparatus able to record pressure changes in Warburg vessels with a strain gauge as detector. Kok (89) constructed a sensitive recording micro-Warburg. The liquid in its manometer capillary was in connection with the liquid in the vessel, and the level of the manometer was kept constant by a pressure regulating servo-mechanism using a photomultiplier as detector. Spruit (56) used a polarographic method for the detection of hydrogen produced upon illumination of an anaerobic suspension of *Chlorella*.

Chloroplast reactions.—The rate of the Hill reaction is sometimes increased by the addition of substances which may act as catalysts. The degree of stimulation depends on the kind and concentration of reductant, on the conditions of the chloroplasts, and on light intensity.

Bradley & Calvin (90) found that thioctic acid stimulated the Hill reaction with quinone as oxidant, but only at high light intensity, low quinone concentration, and high algal density. Since it was inactive or inhibitory under other conditions, the results were considered inconclusive [cf. also Lumry *et al.* (19)]. Wessels (60, p. 56) observed that chloroplasts lost their activity in quinone reduction by frequent washing, but regained activity by addition of washing liquid. Punnett (91) found that yeast extract stimulated the Hill reaction of chloroplast fragments with ferricyanide as oxidant, mainly at high light intensity. Recently he observed (92) at saturating intensity, with 2,6-dichlorophenol indophenol as oxidant, that whole chloroplasts were more stable in glucose while fragmented ones were more stable in potassium chloride solution. Extending previous experiments, Davenport & Hill (93) purified the protein factor from leaves catalyzing reduction of methaemoglobin by illuminated chloroplasts. They found that the factor also stimulated the reduction of added cytochrome-*c*.

Experiments with oxygen isotopes by Mehler, Brown & Good (94, 95),

confirmed Mehler's hypothesis (96) that oxygen in the presence of ethanol and catalase reacts as a Hill reagent [cf. also Good & Hill (97)]. Mehler also found a photooxidation (by oxygen) of ascorbic acid, which was catalyzed by quinone (98), but more efficiently by 2,6-dichlorophenol indophenol [Vernon & Kamen (99)]. This reaction was also observed for extracts of *Rhodospirillum rubrum* (99). The fact that the photo-oxidation of ascorbic acid was not inhibited by the inhibitors of the Hill reaction tested (98, 100) or by heating to 55°C. (99) suggested to Wessels (100) that this reaction was a nonenzymatic reaction, different from the Hill reaction. It perhaps was a combination of excited chlorophyll with oxygen followed by transfer of oxygen to the indophenol-ascorbate system. This type of reaction was observed by Schenck for certain dye-acceptor systems [cf. (19, 100)]. Support for such an interpretation was Wessels' finding (100) that a chlorophyll *a* solution in ethanol photo-oxidized ascorbate in the presence of the indophenol dye.

We mentioned in the section on carbon dioxide reactions the experiments of Whatley *et al.* (11), in which CO_2 fixing activity of chloroplasts could be restored by adding extracted components. These experiments are an important step towards "solubilisation" of the system able to carry out the complete photosynthetic reactions. According to Thomas & Haans (101), fragments of the lamellated chloroplasts of *Spirogyra* are able to fix carbon dioxide with the simultaneous production of oxygen.

Light absorption.—The specific extinction spectra were determined for bacteriochlorophyll and for ethyl chlorophyllides *a* and *b* and their pheophorbides [Holt and Jacobs (25)]. Jacobs *et al.* (103) were able to obtain chlorophyll in the solid state, without changing its spectral properties, by precipitation in the presence of water.

The absorption spectrum of a suspension of photosynthesizing cells is distorted with respect to that of a solution containing the same absorbing substance. One cause of distortion is the scattering. Shibata *et al.* (104) attempted to correct for this distortion by placing a piece of oil-impregnated filter paper immediately behind the absorption vessel. Light rays, scattered by the suspension over wide angles, are "caught" by the paper, which decreases the apparent scattering. However, the measured extinction is higher than that of a corresponding solution, in which the rays traverse a shorter distance. Barer (105) used a theoretically more satisfactory method. He was able to make the refractive index of the solution roughly equal to that of the organisms, which minimized scattering. Another not generally recognized but generally present, distortion is caused by the concentration of the pigments in strongly absorbing particles. A method was developed to correct for this distortion, which makes possible quantitative analysis of absorption spectra of cells in terms of the pigments present [Duysens (106)]. Latimer cf. 1) observed increased scattering at wavelengths just beyond the absorption maxima in *Chlorella.*

Absorption spectrophotometry has been one of the most important methods in the study of oxidation-reduction catalysts in colorless cells and

their extracts. The feasibility of similar studies for photosynthetic organism was demonstrated with an apparatus for measuring very small changes in light absorption upon illumination [Duysens (35)]. These changes were measured at various wavelengths and, in general, found to be reversible upon darkening. With increasing light intensity a saturation of the change in absorption was reached. The maximum change in optical density was of the order of 1 per cent of that of chlorophyll *a* or bacteriochlorophyll in the long-wavelength maximum. The difference between the steady state absorption in light and darkness is plotted as a function of the wavelength, and this "difference spectrum" equals the sum of the difference spectra of substances, which change their absorption spectrum upon onset of photosynthesis. Also the time course of the change can be followed. More recently Chance *et al.* (107) described a similar apparatus, which was used for measuring changes in the absorption spectrum upon illumination of carbon monoxide-cytochrome complexes in colorless bacteria. They used the same apparatus later for measuring changes in absorption in purple bacteria (67). French *et al.* (108) reported on the performance of a spectrophotometer, which plots the first derivative of transmission with respect to wavelength. This apparatus seems particularly useful for detecting sharp absorption bands on a continuous background of high absorption.

The number of applications of the method for measuring small absorption changes upon illumination of photosynthetic organisms is rapidly increasing. We shall discuss results with *Chlorella*. The difference spectrum of *Chlorella* shows a negative peak at 555 mμ [Lundegardh (109)] and one at 420 mμ (110). The decreases in absorption at 420 and 555 are caused by oxidation of a cytochrome, presumably *f* (58), and the increase around 340 mμ (58) possibly by reduction of pyridine nucleotide. Furthermore, the difference spectrum of *Chlorella* shows a pronounced maximum at 520 mμ and a negative maximum at 480 mμ (110) caused by an unknown substance. This substance does not seem to be connected with oxygen evolution, since the changes in absorption are strongly influenced by iodoacetate (111), which does not inhibit oxygen production in the Hill reaction. No changes, comparable to those at 520 mμ were observed in the red region (111), a fact which argues against chlorophyll *a* as the cause of the change at 520 mμ. Witt (112, 113) measured with an oscillograph rapid changes in absorption of *Chlorella*, brought about by light flashes. He did not give a difference spectrum, but reported an increase at 515 mμ and a decrease in absorption at 475 mμ, at about the same wavelengths as had been found by Duysens with a slower method and the use of continuous light. According to Witt the increase in absorption occurs at room temperature within 4×10^{-5} sec. and the decrease after the flash in about 10^{-2} sec.

Pronounced short-lived spectral changes were observed by Livingston (28) with a chlorophyll solution illuminated by a flash. The changes were different from those observed in *Chlorella*. The spectrum is different, the changes in *Chlorella* are about hundred times smaller, and the *Chlorella*

changes take place in the presence of air while the changes in the chlorophyll solution occur only in the absence of oxygen.

Fluorescence and luminescence.—French (38) gave a review of experiments on spectral fluorescence carried out in his laboratories. Fluorescence spectra of highly colored tissue are distorted by selective reabsorption of the fluorescent light. For leaves of land plants this distortion is often much greater than is to be expected by selective absorption alone. Virgin (114) demonstrated that this effect was attributable to excessive scattering of the fluorescent light. The scattering was caused by air-filled intracellular spaces. During illumination the scattering varied, which caused a change in the fluorescence spectrum. Infiltration of the leaves with water minimizes these effects and makes it possible to use leaves of land plants also for the study of fluorescence phenomena.

Virgin (115) also used the fluorescence spectrum as a means for detecting protochlorophyll and chlorophyll *a* and was able to follow in this way the light-induced transformation of protochlorophyll into chlorophyll. With periodic flashes of 3 msec., and a dark time of 13 msec., the yield was the same as with continuous light. This indicates that no slow dark reaction is involved in the transformation of protochlorophyll, which agrees with the observation of Smith & Benitez (102) that the transformation still occurs at −70°C.

Goedheer (116) measured the polarisation spectrum of the fluorescence of chlorophyll *a*, bacteriochlorophyll, and their pheophytins in a viscous solvent, using polarized exciting light. The results were found to be in reasonable agreement with the calculations of Longuet Higgins *et al.* (117) and Platt (118) on tetrahydroporphin and porphin, and enabled a plausible allocation of the direction of the electronic vibration with respect to the plane of the conjugated ring system. Goedheer (119) also studied the orientation of pigment molecules with respect to the large plate-shaped lamellated chloroplasts of *Mougeotia*. If the planes of the chlorophyll molecules are orientated parallel to the plane of the chloroplasts, one may expect a smaller absorption for light polarised perpendicular to the chloroplast plane than for light polarised parallel to it. Such a difference in absorption was indeed found. This phenomenon is correlated with birefringence which could be measured with greater precision. Goedheer's conclusion was that the planes of the chlorophyll molecules were partly oriented parallel to the lamella. The carotenoids show a weak orientation, also parallel to the plane of the lamellae.

Arnold (120) found that the "delayed light" emitted by chlorophyll *a* in *Chlorella* a short time after interruption of illumination is saturated at an intensity much lower than that needed to saturate photosynthesis. This seems to indicate that the substance responsible for the delayed light is not in the main chains of reactions leading to carbon dioxide fixation and oxygen production. Arnold further estimated that saturation for light flashes is just obtained at an intensity at which one out of 200 to 600 chlorophyll molecules absorb one quantum, which indicates that the substance is present in an amount which is small compared to that of chlorophyll.

Strehler & Cormier (121) had found that light emission of acetone powders of luminescent bacteria were stimulated by a long-chain aldehyde. Ferrari *et al.* (122) found the same to be true for extracts from illuminated plants. There is possibly a relation between the complexes active in the primary reaction discussed in a preceding section, the substance responsible for spectral changes at 520 mμ in *Chlorella*, and the substance causing the delayed light emission, but it is still too early to draw definite conclusions.

Griffiths *et al.* (123) obtained by ultraviolet irradiation a green blue mutant of the athiorhodacea *Rhodopseudomonas spheroides*. The mutant contained bacteriochlorophyll but, although no carotenoids were present, was able to photosynthesize under anaerobic conditions. Apparently carotenoids, in bulk at least, are not essential for photosynthesis. When, air was present, light killed the mutant, but not the wild strain. Carotenoids may protect, as Griffiths *et al.*, concluded, illuminated cells against damage in presence of air, but this interpretation is not the only one, since the absence of carotenoids and sensitivity to oxygen may have a common origin.

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NITROGEN FIXATION BY PHOTOSYNTHETIC ORGANISMS[1]

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Introduction

Virtually unlimited amounts of the principal constituent elements of living matter are present on earth, but organic production is primarily dependent on the conversion of two of these elements, carbon and nitrogen, from the forms in which they are most abundant to forms which can be used directly for synthesis of protoplasm. The abilities to carry out these two major transformations, the photosynthesis of organic substances from carbon dioxide and the "fixation" of elementary nitrogen, are restricted to certain groups of organisms and do not necessarily occur together. Nevertheless, a few species are able to carry out both photosynthesis and nitrogen fixation. The study of these self-sufficient organisms is of particular interest for comparative biochemistry and for the understanding of the factors controlling the productivity of soils and waters.

General aspects of the physiology and biochemistry of nitrogen fixation have been considered recently in reviews by Virtanen (106), Wilson (115, 116), and Wilson & Burris (117). There appears to have been no review hitherto specifically concerned with nitrogen fixation by photosynthetic organisms but other aspects of the physiology of the organisms involved have received consideration recently (41, 100). Here, attention will be confined to nitrogen fixation in photosynthetic organisms, the interrelations between photosynthesis and nitrogen fixation, and the part which organisms able to carry out both these processes play in natural economy. Nitrogen fixation by nonphotosynthetic organisms in symbiotic association with green plants will not be dealt with except in so far as it bears on the problem of the relationships between photosynthesis and nitrogen fixation. On the subject of symbiotic nitrogen fixation there are Wilson's classic monograph (114) and more recent reviews by Virtanen (105) and Allen & Allen (5).

The Occurrence of Nitrogen Fixation in Photosynthetic Organisms

Blue-green algae.—Blue-green algae (Myxophyceae or Cyanophyceae) were among the first organisms to attract attention as fixers of nitrogen, being studied, for example, by Frank (43) and Beijerinck (13) towards the close of the nineteenth century. Nevertheless, because of the difficulty of isolating these organisms in bacteria-free condition, definite proof that any species possessed the property was not forthcoming until the work of

[1] The survey of the literature pertaining to this review was completed in October, 1955.

Drewes was published in 1928 (32). Since then, the list of algae known to fix nitrogen has steadily lengthened, and now there is trustworthy evidence that more than 20 species, about half of those of which the nitrogen nutrition has been critically examined, possess the property (41). Usually, proof of nitrogen fixation has consisted of demonstration by the Kjeldahl method of an increase in total combined nitrogen in cultures made in a medium without added nitrogenous substances, but the more convincing method using N^{15} as a tracer has also been used (22, 23, 113).

The distribution of the capacity to fix nitrogen among the various groups of blue-green algae has been considered by Fogg & Wolfe (41). However, certain additions and alterations to the lists of species given in their paper are now necessary. The investigations of Hérisset (55) with pure cultures of *Nostoc commune* show that this species must be included with the nitrogen-fixers. Douin (30) has suggested that the algae studied by Winter (118) were strains of *Anabaena cycadeae* not of *Nostoc punctiforme*. *Anacystis nidulans* and a strain of *Anabaena variabilis* have been found not to fix nitrogen (63). Other strains of *A. variabilis* are able to fix nitrogen (41) and, while it is not at all unlikely that nonnitrogen-fixing strains of this or any other species should exist, it is possible that this particular alga has been incorrectly identified; its filaments tend to break up into short lengths and in culture at University College, London, it has not been observed to form heterocysts, in both of which respects it is exceptional for an *Anabaena*.

The principal genera known to contain nitrogen-fixing species are *Anabaena*, *Nostoc* and *Cylindrospermum*, all of which belong to the Nostocaceae. Two other families included in the Nostocales, the Rivulariaceae and Scytonemataceae, have been shown to include nitrogen-fixing species but it seems that the capacity is lacking in another family of this order, the Oscillatoriaceae (41). Very few species of other orders have been examined, but *Mastigocladus laminosus*, a thermal alga belonging to the Stigonematales, has been shown to be nitrogen-fixing (38), whereas, of seven species of Chroococcales, unicellular blue-green algae, that have been examined, none have been found to possess the property (41). It is noteworthy that all of the species known to be nitrogen-fixing possess the enigmatical structures known as heterocysts, whereas those unable to assimilate elementary nitrogen are for the most part nonheterocystous. The association of these two characters may perhaps imply a phylogenetic relationship between the species possessing them which is not apparent in the schemes of classification in use at present.

Photosynthetic bacteria.—Photosynthetic bacteria had been studied in laboratory culture for some 60 years before their capacity for nitrogen fixation was detected. The observation that *Rhodospirillum rubrum*, which is able to produce hydrogen in the light when supplied with certain organic acids, does not do so in the presence of ammonium salts or elementary nitrogen, led Gest & Kamen (48), who had in mind the finding of Lee & Wilson (66) that hydrogenase activity and nitrogen fixation are correlated in *Azotobacter*, to suspect that this nonsulphur purple bacterium might be able to fix nitrogen. Proof that *R. rubrum* can indeed fix nitrogen was obtained

using N^{15} as a tracer (60), and confirmation, both by the tracer method and Kjeldahl analysis was supplied by Lindstrom, Burris & Wilson (69). Other photosynthetic bacteria were then found to possess the property, the species for which there is now published evidence of nitrogen fixation being as follows: *Chromatium sp.* (69, 72, 79) in the Thiorhodaceae; *Chlorobium* (*Chlorobacterium*) *thiosulfatophilum* (64, 69, 72) in the Chlorobacteriaceae; *Rhodospirillum rubrum* (60, 69), *Rhodopseudomonas sp.*, *R. capsulatus*, *R. gelatinosa*, *R. sphaeroides*, *R. palustris* (70) and *Rhodomicrobium vannielii* (70, 72) in the Athiorhodaceae. Of 20 strains of *Rhodopseudomonas* tested for nitrogen fixation, 19 gave positive results (70), and it would appear that the property is one generally possessed by photosynthetic bacteria.

Algae other than Myxophyceae, and higher plants.—Very few algae have been examined critically from this point of view, and, with the instance of the photosynthetic bacteria before us, it would be unwise to assert that no algae other than blue-greens are able to fix nitrogen. Nevertheless it seems that species of Chlorophyceae are unable to assimilate elementary nitrogen since several workers have reported failure to demonstrate this [for references to the older literature see (32)], and evidence to the contrary is unsatisfactory. Thus evidence apparently showing that *Chlorella* spp. and various other green algae can fix nitrogen (110) has been shown to have been obtained by uncritical use of the Kjeldahl technique (19). Assertions that various chlorococcoid freshwater algae and a marine *Enteromorpha* are nitrogen-fixing (75, 76) can be discounted because the cultures used in obtaining the evidence contained bacteria. More serious consideration must be given to a tentative report of nitrogen fixation in pure cultures of *Chlorella pyrenoidosa* in which gains in total nitrogen were demonstrated by Spoehr & Milner using the absolute method of Dumas (94). However, it seems probable that here the gains observed arose by absorption of traces of ammonia and oxides of nitrogen from the air (26). Since *Scenedesmus* develops hydrogenase activity on incubation under anaerobic conditions it was suspected that it might be able to fix nitrogen but tests using N^{15} as a tracer gave no evidence for this (69). There are, in fact, rather important differences between the hydrogenases of *Scenedesmus* and of the photosynthetic bacteria (50). Algae belonging to other classes, e.g., Bacillariophyceae, Phaeophyceae, and Rhodophyceae, do not appear to have been tested for nitrogen fixation, but there is little to suggest that any of them possess the property.

Wilson (114) has discussed critically various reports of nitrogen fixation by higher plants and has shown them to be without satisfactory foundation. Experiments using radioactive N^{13}, which has a half-life of 10.5 min., as a tracer seemed to afford evidence of nitrogen fixation by barley (87), but this could not be confirmed using N^{15}, and the reported uptake of the radioactive isotope was considered to arise from nonspecific surface adsorption (21). Tests for nitrogen fixation using N^{15} as a tracer with germinating legume seeds, uninoculated legumes, and nonleguminous plants have yielded consistently negative results (22).

From the evidence at present available it thus appears that organisms

possessing capacities both for nitrogen fixation and for photosynthesis are confined to the Myxophyceae and photosynthetic bacteria. There does not appear to be any close relationship between these two groups (85), but, nevertheless, they possess two striking features in common: firstly, each contains a high proportion of nitrogen-fixing species; secondly, each possesses characteristics suggesting that it appeared comparatively early in the course of development of life upon earth. Such characteristics are the relative simplicity of cell structure, cosmopolitan distribution, and types of metabolism which may be considered as primarily adapted to primitive earth conditions. Hutchinson (quoted in 115) has pointed out that, although ammonia probably occurred in substantial amounts under the conditions prevailing at the time when life first appeared upon earth, oxidation to nitrate and the appearance of denitrifying organisms would result in rapid depletion of this stock of combined nitrogen. It thus seems that organisms capable of transforming elementary nitrogen back into the combined state must have appeared at an early date or life would have become extinct. The blue-green algae and photosynthetic bacteria evidently underwent their greatest development at a stage when the shortage of combined nitrogen was becoming acute. Higher plants and animals appear to have evolved from nonnitrogen-fixing stocks but the frequency with which symbioses have arisen between higher photosynthetic plants and nitrogen-fixing micro-organisms, both photosynthetic and heterotrophic, testifies to the biological advantage of the combination found in the blue-green algae and photosynthetic bacteria.

General Physiology of Growth and Nitrogen Fixation in Blue-Green Algae

There appears to have been no recent review of the general physiology of blue-green algae, but methods of isolation and culture have been discussed in several publications (2, 46, 47, 63, 84). All the nitrogen-fixing blue-green algae studied so far are capable of phototrophic growth in purely mineral media with the exception of an endophytic strain of *Nostoc punctiforme* [or *Anabaena cycadeae* (see 30)] which Winter (118) found to be scarcely capable of growth and incapable of nitrogen fixation in inorganic medium in the light. This alga was able to grow heterotrophically in the dark if supplied with fructose and fixed nitrogen under these circumstances. A curious finding reported by Winter is that, although the alga grew in the dark on glucose or mannose if combined nitrogen were present, it only fixed nitrogen when the carbon source was fructose. Douin (30), on the other hand, found *A. cycadeae* to grow in the absence of supplied combined nitrogen if provided with glucose or sucrose. Allison's strain of *Nostoc muscorum* (*N. muscorum* A) can also grow and fix nitrogen slowly in the dark if provided with glucose or sucrose (7, 63) but other nitrogen-fixers, such as *Anabaena cylindrica* (41) and Gerloff's strain of *N. muscorum* (*N. muscorum* G) (63), appear to be obligate phototrophs. *Nostoc muscorum* (strain not stated) is able to assimilate acetate photosynthetically without first oxidizing it to carbon dioxide (9)

but the carbon of formate or urea is only assimilated by this alga following oxidation or hydrolysis, respectively, to carbon dioxide (10).

Although there have been a few excellent studies on the kinetics of photosynthesis by blue-green algae, especially in relation to the role of accessory pigments (14), the species used have not been nitrogen-fixing ones. The light intensities required to saturate photosynthesis are of the order of 260 foot-candles for *N. muscorum* G (63) and at least 310 foot-candles for *A. cylindrica* (37). Either fluorescent or tungsten illumination will support maximum growth of *N. muscorum* G (63).

Blue-green algae are able to assimilate nitrogen supplied as nitrate or ammonia or in organic form and nitrogen fixation is then suppressed to an extent depending on the availability of the form of nitrogen supplied. Since this subject was reviewed by Fogg & Wolfe (41), relative growth constants (k) for *N. muscorum* G supplied with various nitrogen sources have been published by Kratz & Myers (63). At 25°C., k, in log units per day with carbon dioxide and light saturation, of growth was 0.37, 0.50, 0.48 and 0.54, respectively, when the alga was supplied with elementary nitrogen, nitrate, ammonia, and urea. Growth rates on glycine or casein hydrolysate were unstable. Although *N. muscorum* G thus grows more slowly when fixing nitrogen than when assimilating nitrate, *A. cylindrica* has been found to grow equally rapidly with either source (3, 37). It is possible that in the experiments of Kratz & Myers molybdenum, which was supplied in a concentration of 0.01 p.p.m., was limiting growth on elementary nitrogen. However, Magee & Burris (74), using the same strain supplied with an apparently adequate amount of molybdenum, found nitrogen assimilation to be more rapid when the source was nitrate as compared with the element. Both *A. cylindrica* (41) and *N. muscorum* G (74) require a period of several hours for adaptation to nitrate after previous growth on elementary nitrogen. Nitrite has been found in the medium following supply of nitrate to these algae but hydroxylamine has not been detected (74, 120). When N^{15}-labelled nitrate was supplied to *N. muscorum* G and the distribution of tracer determined 90 min. later, the hydrolysate ammonia was found by Magee & Burris to contain a higher concentration of tracer than the glutamic acid (74). High N^{15} concentrations were also found in cytosine, guanine, uracil, xanthine, and an unknown compound. Thus under the particular conditions used there was a more rapid synthesis of purines and pyrimidines relative to synthesis of amino acids when nitrate rather than elementary nitrogen was supplied. It would be desirable to determine whether this is a characteristic of nitrate assimilation under other physiological conditions.

Other aspects of inorganic nutrition have received some attention. Molybdenum was found by Bortels to be essential for the growth of seven species of blue-green algae in a medium without supplied combined nitrogen but a requirement for this element in the presence of nitrate or ammonium salts was not investigated (16). Wolfe, however, found molybdenum to be essential for the growth of *A. cylindrica* on nitrate, saturation being reached

at 0.1 p.p.m. whereas more than 0.2 p.p.m. were required to give optimum growth on elementary nitrogen (119). Growth of this alga took place in the absence of these relatively high concentrations of molybdenum if ammonia was supplied but the question remains open whether or not growth with this nitrogen source is possible in the complete absence of molybdenum. Allen (2) found sodium to be needed for good growth of various blue-green algae and the necessity of this element for the growth of *N. muscorum* G (63) and *A. cylindrica* (4) has been demonstrated in carefully controlled experiments. For *A. cylindrica* a sodium concentration of 5 p.p.m. or higher is required for optimum growth and this element cannot be replaced by potassium, lithium, rubidium, or caesium. Sodium cannot, however, replace potassium in the nutrition of *A. cylindrica* (4). The sodium/potassium ratio seems to have no important effect on growth (63). Boron has been found essential for maximum growth of *N. muscorum* G, the optimum concentration being 0.1 p.p.m. and concentrations greater than 1.0 p.p.m. being toxic (35). Experiments with *N. muscorum* G and *Calothrix parietina*, as well as with non-nitrogen-fixing species, have shown cobalt to be an essential element for blue-green algae. At least 0.004 p.p.m. of cobalt is needed for optimum growth of *N. muscorum* G unless the element is presented in the form of cobalamin, when much lower concentrations suffice. Cobalt appears to have no specific function in the nitrogen fixation process (56).

No requirement for organic growth factors appears to have been demonstrated for a nitrogen-fixing blue-green alga. It may be noted, however, that the nonnitrogen-fixing species, *Phormidium persicinum*, has a requirement for purine, thiamine, and possibly other vitamins (86).

Although it has been stated frequently that blue-green algae grow best under slightly alkaline conditions few precise determinations of the effects of the reaction of the medium upon growth have been made. According to Allison and co-workers (6, 7) *N. muscorum* A will not grow in a medium without supplied combined nitrogen outside the range pH 5.7 to 9.0, growth being best between pH 7.0 and 8.5 and decreasing rather markedly below pH 6.5. Nevertheless, Walp & Schopbach (109) found *N. muscorum* A to grow at reactions as acid as pH 4.0 to 4.9 in nitrate-free medium. Such differences may well depend on different ionic ratios in the media used. "Growth" in these investigations was taken as final yield. More critical determinations have been carried out by Kratz & Myers (63) who found for *N. muscorum* G that growth in a nitrate medium occurred between pH 6.9 and 9.0, the relative growth constant changing very little with variation in reaction within this range. No specific effect of hydrogen ion on nitrogen fixation, such as occurs in *Azotobacter* (20), has been reported for a blue-green alga. Walp & Schopbach (109) found no differences in growth of *N. muscorum* A in media of pH 5.0 depending on whether nitrate or elementary nitrogen was assimilated but their method of estimating growth had no great degree of sensitivity.

A little information about the effects of temperature is available. *N.*

muscorum A has been reported as growing at temperatures up to 40 to 44°C. with an optimum at 28 to 30°C. (6), but Kratz & Myers (63) found that the same strain failed to grow at 41°C. For this alga and for *A. cylindrica* growth was better at 32.5°C. than at 25°C. For *N. muscorum* G the relative growth factor, k, reached a maximum value of 0.86 at 32.5°C. and was still high at 35°C. (63). *Mastigocladus laminosus* is reported to grow in hot springs at temperatures of *c.* 50°C. (73, 107) and to grow poorly at less than 40°C. (107), but some strains, at least, have been grown satisfactorily at physiologically more normal temperatures (38, 73).

Recent work (3, 63) makes it clear that the general impression given by earlier literature, that blue-green algae are slow growing, is incorrect. The minimum generation time for *N. muscorum* G appears to be just over 8 hr. and the existence of a nonnitrogen-fixing blue-green alga, *Anacystis nidulans*, with a generation time of just over 2 hr. at 41°C., the highest growth rate reported for an alga, suggests that faster growing nitrogen-fixing species than *N. muscorum* G may yet be found (63).

As in most organisms possessing the property, nitrogen fixation in blue-green algae rather closely parallels growth. Thus, compared with other algae, the total nitrogen content of which may vary within wide limits, *A. cylindrica* shows little variation in nitrogen content during its growth cycle in culture (26, 36). There is, however, considerable variation in the proportion of nitrogen fixed appearing in extracellular form. The liberation from the cells of substantial amounts of soluble nitrogenous substances in healthy cultures of nitrogen-fixing blue-green algae has been recorded many times (8, 27, 38, 53, 74, 111) and the phenomenon has been investigated in detail for *A. cylindrica* (39). In this species, and probably in others also, the substances concerned are principally polypeptides (39, 74), free amino acids being present only in minute amounts (39, 74, 111) except in cultures of *Calothrix brevissima*, in which appreciable amounts of aspartic acid, glutamic acid, and alanine, have been found (111). In one sample of extracellular polypeptide from *A. cylindrica* the principal component amino acids were found to be serine and threonine with smaller amounts of glutamic acid, glycine, and tyrosine, and traces of alanine, valine, and leucine (39). The liberation of extracellular polypeptide is not attributable to autolysis and appears invariably to accompany growth. The relative amounts of extracellular nitrogenous substances produced vary with the cultural conditions being, for example, increased in the later stages of growth by deficiency of iron (39). The low rates of excretion recently reported for *N. muscorum* G (74) and for *A. cylindrica* (3) may perhaps be attributed to better supply of trace elements than was achieved in the experiments of Fogg (39) and earlier workers. The extracellular nitrogenous products of *A. cylindrica* do not appear to include any appreciable proportion of substances specifically associated with the fixation process since the relative amount and composition of the fraction remains much the same when the alga is cultivated on nitrate or ammonium nitrogen as when only elementary nitrogen is supplied

(39). Because of their capacity to form complexes with various ions, extracellular polypeptides have important effects on the growth of the alga in culture and probably also in natural environments (40).

General Physiology of Growth and Nitrogen Fixation in Photosynthetic Bacteria

For the assessment of the importance of photosynthetic bacteria as nitrogen-fixers it is necessary in the first place to bear in mind their more outstanding nutritional characteristics. These are as follows:

Thiorhodaceae: Obligately anaerobic or micro-aerophilic; growth occurs in the light using sulphide, hydrogen, or organic substances such as lower fatty acids, as hydrogen donors; organic growth factors not required; no convincing evidence for growth in the dark has been published (97, 98).

Chlorobacteriaceae: Strict anaerobes; growth occurs in the light, utilizing reduced sulphur compounds or hydrogen as hydrogen donors; organic growth factors not required; growth in the dark does not occur (64, 65).

Athiorhodaceae: Growth occurs anaerobically in the light using organic substrates, sulphide, or hydrogen, as hydrogen donors, or aerobically in the dark on organic substrates (33, 98, 99); organic growth factors essential (except for *Rhodomicrobium vannielii*), one or more of the following being required both for aerobic and anaerobic growth; thiamine, nicotinic acid, biotin, *p*-aminobenzoic acid (58).

A detailed review of the physiology and biochemistry of these organisms has recently been made by van Niel (100), and a more general account has been given by Lees (67), so that here attention may be confined to information more directly concerning their capacity for nitrogen fixation. However, an important line of research which has developed recently, namely the investigations of the cytochromes of photosynthetic bacteria made by Kamen & Vernon (61, 62, 102, 103, 104), should be noted although so far it does not appear to have any direct bearing on the problems of nitrogen fixation.

Various species of Athiorhodaceae (48, 49, 50, 59) and *Chromatium* sp. among the Thiorhodaceae (17) have been found to evolve elementary hydrogen in the light, this being the property the investigation of which led to the discovery of their nitrogen-fixing powers. At first, hydrogen production was thought to be dependent on exogenous supplies of particular organic acids, malic, fumaric, and succinic acids being effective for *Rhodospirillum rubrum*, for example. Later, it was found that the presence of a minimum concentration of carbon dioxide or bicarbonate was sometimes critical for the photoproduction of hydrogen and re-investigation showed that the process is endogenous and accelerated by the supply of almost any readily metabolized substrate (91). Carbon dioxide is evolved simultaneously with hydrogen (48), the relative amounts of the two gases evolved for a given amount of substrate consumed varying with the nature of the substrate in no readily interpretable manner (91). The stoichiometry of photoproduction of hydro-

gen has been investigated in detail for *R. rubrum* supplied with malate by Bregoff & Kamen (18). In the absence of an exogenous nitrogen supply one mole of hydrogen and one mole of carbon dioxide was evolved per mole of malate consumed. The mechanisms of these phenomena, which are not fully understood, have been discussed by Kamen (59), Bregoff & Kamen (18), and van Niel (100).

Photoproduction of hydrogen is completely suppressed in the presence of ammonium chloride or elementary nitrogen (17, 18, 48, 59, 69, 79, 91). Morita *et al.*, however, found the inhibition of the photoproduction of hydrogen by elementary nitrogen or ammonia in *Rhodobacillus palustris* to be incomplete (77). The nitrogen source is utilized by the organism, and photoproduction of hydrogen is resumed when it has disappeared (59, 18). No constant ratio has been found between the amounts of ammonia assimilated and hydrogen which would have been expected to be produced from the malate utilized (18). Growth and photoproduction of hydrogen by *R. rubrum* can occur in the presence of certain amino acids, e.g., L-glutamic, L-aspartic, L-arginine, L-proline, and L-leucine. Certain other amino acids are growth promoting for this bacterium but inhibit hydrogen production, free ammonia being formed from them (18). The photoreduction of carbon dioxide by hydrogen is not inhibited by the supply of ammonia or elementary nitrogen. The evolution of carbon dioxide accompanying that of hydrogen may, however, be partially suppressed by ammonium chloride and, to a lesser extent, by elementary nitrogen (91). *R. rubrum* was found to produce one mole of carbon dioxide for each mole of malate dissimilated in the presence of ammonium chloride (18). A hypothesis explaining the suppression of the photoproduction of hydrogen by nitrogen has been put forward by Kamen (59) and elaborated by Newton & Wilson (79). These latter workers suggest that the compound involved in hydrogen production is closely related to the carbon compound, α-ketoglutaric acid perhaps, which acts as the acceptor for the ammonia supplied or produced by nitrogen fixation. When the bacterium is supplied with a nitrogen source this compound is consumed in this way and none is available for hydrogen production. Hydrogen production by growing cells of *R. rubrum* has been observed (50), but the nitrogen was supplied in organic form in these cultures so that carbon skeletons were unnecessary for its assimilation and photosynthesis may be judged to have been producing products in excess of cell requirements. The photoproduction of hydrogen may thus be regarded as an "idling" of the photosynthetic mechanism which ceases when the products of photosynthesis are used for synthesis of nitrogenous compounds. This is in agreement with the fact that photoproduction of hydrogen by anaerobically adapted *Scenedesmus*, which cannot fix nitrogen, is not suppressed by elementary nitrogen (59). It is also in agreement with the finding that although the presence of nitrate in the growth medium has been found to decrease the hydrogenase activity of the nonnitrogen-fixing facultative autotroph *Hydrogenomonas facilis*, nitrate, nitrite, nitrous oxide, and hydroxylamine were found to have no direct inhibiting effect

on hydrogenase activity as determined by methylene blue assay (11). Assimilation of elementary nitrogen by *R. rubrum* is inhibited in the presence of ammonia (50, 60).

Fixation of nitrogen by photosynthetic bacteria has been found to be much more rapid in the light than in the dark (60, 69, 71). The fixation rate of *R. rubrum* maintained aerobically (71) or anaerobically (50) in the dark was only about 1 per cent of that achieved when photoreduction was taking place. This relationship is discussed further in the next section.

There appear to be no quantitative data published regarding the effects on photosynthetic bacteria of other factors, such as reaction or the presence of molybednum or other trace elements, which might be expected to have specific effects on their nitrogen fixation. Growth has usually been found to be best at neutral or slightly alkaline reactions (64, 99.) The relative rates of fixation reported for *Rhodospirillum* spp. (60, 69, 70), *Chlorobium* (*Chlorobacterium*) *sp.*, and *Chromatium sp.* (72), are all rather low, corresponding to a doubling of cell nitrogen in three to eight days.

The Mechanism of Nitrogen Fixation in Photosynthetic Organisms

The enzyme system fixing nitrogen has so far been examined only for *N. muscorum* A among the photosynthetic nitrogen-fixers. In this organism, at least, its properties are similar to those of the *Azotobacter* and *Rhizobium*-legume enzymes (24). The partial pressure of nitrogen giving half the maximum rate of fixation was found by Burris & Wilson to be of the order of 0.02 atm. for *Nostoc* as compared with 0.02 and 0.05 atm. for *Azotobacter* and *Trifolium pratense* respectively. Like those of other nitrogen-fixing organisms, the *Nostoc* nitrogenase is specifically inhibited by hydrogen and by carbon monoxide. One difference is that in *Nostoc* the degree of inhibition by carbon monoxide appears to depend on pN_2, whereas in *Azotobacter* and *Trifolium* the inhibition is noncompetitive. Nitrogen fixation by photosynthetic bacteria appears not to be inhibitied by hydrogen, a state of affairs similar to that in *Clostridium* (78).

N. muscorum (strain not stated), *Cylindrospermum* sp. (44), and *A. cylindrica* (120) do not appear to possess hydrogenase systems. Thus, on present evidence, these blue-green algae show none of the correlation between hydrogenase activity and nitrogen fixation which is to be seen in *Azotobacter* (51) and the photosynthetic bacteria.

The path of nitrogen in nitrogen fixation has been investigated both in photosynthetic bacteria by Wall *et al.* (108) and in *N. muscorum* G by Magee & Burris (74) using N^{15} as a tracer. Suspensions of *R. rubrum*, *Chlorobium* (*Chlorobacterium*) sp., and *Chromatium* sp., were supplied N^{15}-enriched elementary nitrogen for short periods, then the cells were recovered, hydrolysed, and the amino acids and ammonia from the hydrolysates were isolated by chromatographic procedures. Determination of the distribution of N^{15} showed in every instance the highest concentration to be in glutamic acid, the ammonia fraction always having the next highest concentration. A

similar distribution of tracer following supply of N^{15}-labelled ammonia was found using *Chromatium* sp. (108). In *Nostoc*, too, the highest concentrations of tracer were found in glutamic acid and ammonia when either labelled ammonia or the labelled element was supplied (74). The results of these tracer experiments are similar to those of comparable experiments carried out with other nitrogen-fixing agents, e.g., *Azotobacter* and the nodules of leguminous plants, and indicate that ammonia is the key intermediate in fixation, the main path of entry into metabolism being by amination of α-ketoglutaric acid (116).

The specific requirement for relatively high concentrations of molybdenum in nitrate assimilation and nitrogen fixation by blue-green algae suggests that these two processes have something in common. The work of Wolfe (119, 120), however, indicates that this common feature may be little more than a requirement for hydrogen donors extra to that of cells supplied with ammonia. Wolfe has shown that although nitrate is reduced to ammonia in molybdenum-deficient cells of *A. cylindrica*, the assimilated nitrogen accumulates mainly in the ammonia and amide fractions, synthesis of peptides and proteins proceeding very slowly in such cells. This is surprising when it is considered that molybedenum-deficient cells supplied with ammonia as such are capable of normal protein synthesis and growth. Molybdenum-deficient cells are in addition remarkable for showing a rate of endogenous respiration about twice that of normal cells. The supply of fumaric acid enables molybdenum-deficient cells to assimilate nitrate to completion with the production of peptides and proteins. These various facts can be explained on the assumption that the dominant role of molybdenum in *A. cylindrica* under these conditions is the inhibition of phosphatase activity In molybdenum-deficient cells enhanced phosphatase activity may be postulated as being compensated for by an increased rate of production of high energy phosphates by respiration, but, when nitrate rather than ammonia is supplied, hydrogen donors for its reduction are obtained at the expense of respiration so that this balance is upset. Nitrogen fixation, involving as it does the reduction of the element to the ammonia level, will affect the metabolic balance of molybdenum-deficient cells in a qualitatively similar manner to nitrate reduction. If this interpretation is correct, the similar requirement for molybdenum in the two processes does not mean that they have any part of their pathways in common (41). The finding that the activity of purified preparations of *Clostridium* hydrogenase is restored by addition of molybdenum (90) provides yet another possible connexion between hydrogenase and nitrogenase.

The Interrelations of Photosynthesis and Nitrogen Fixation

Nitrogen fixation occurs in micro-organisms representing nearly all nutritional types (115), and there is no tendency for the capacities for nitrogen fixation and photosynthesis to be associated. Clearly the two processes can be independent.

There appear to be no features of photosynthesis in the blue-green algae

or in the photosynthetic bacteria which might be specifically related to their capacity for nitrogen fixation. Their pigment systems show special features (14, 100), but it seems unlikely that these could directly influence nitrogen fixation, and the path of carbon in photosynthesis appears to be essentially the same in these organisms as in green algae and higher plants. The products of short-term photosynthesis in four species of blue-green algae, two of them, *N. muscorum* (strain not stated) and *Nostoc* sp., presumably capable of nitrogen fixation, have been investigated by Norris *et al.* (80). The algae were grown in media containing combined nitrogen, but for the carbon dioxide fixation experiments they were suspended in distilled water, and it seems that nitrogen fixation could have occurred to some extent during the experimental period. The products of short-term photosynthesis were found to be qualitatively the same in all the plants examined except that an unidentified weakly ninhydrin-positive compound was prominent in the blue-green algae but absent or present in small amounts only in a variety of other plants, including green algae and higher plants, which were examined. This compound was, however, found in all four blue-green algae and does not appear to have any specific relation to nitrogen fixation. Stoppani *et al.* (96) have investigated the mechanism of carbon dioxide fixation in *Rhodopseudomonas capsulatus*. These experiments were made in the absence of any source of nitrogen whatsoever. In the light carbon dioxide was assimilated via the phosphoglyceric acid cycle as in other photosynthetic organisms.

The only suggestion of a specific association of the two processes comes from the observations already noted that the extent of fixation by photosynthetic bacteria is considerably greater in the light than in the dark. It is not impossible that nitrogen fixation in these forms should require a specific hydrogen donor, the formation of which is dependent on the photochemical reaction. The condition of obligate phototrophy which obtains in many algae and bacteria seems to demonstrate that processes which in many organisms are light independent may in others become quite dependent on the products of the photochemical process of photosynthesis. Nevertheless there appears to be no unequivocal proof of a specific effect of light on nitrogen fixation in these bacteria. Lindstrom *et al.* (71) found that *R. rubrum* grown heterotrophically and aerobically in the dark and then exposed in the dark to N_2^{15} fixed the tracer at the same level as did photosynthetically grown cells exposed to N_2^{15} in the dark. The bacteria thus appear to have a non-photosynthetic mechanism that can supply at a low level whatever is necessary for nitrogen fixation. A comparison of growth rates on elementary and on combined nitrogen under both dark aerobic and light anaerobic conditions would be desirable to settle this question finally. It is not known whether in the obligately phototrophic blue-green algae nitrogen fixation is strictly confined to periods of illumination. An endophytic species which Winter (118) found to fix nitrogen best during heterotrophic growth, has already been mentioned. Hérisset (55) found that *Nostoc commune* fixed nitrogen in the light whereas in the dark with sucrose no nitrogen fixation could be demon-

strated, but his analytical method was not sufficiently sensitive to detect small amounts of fixation.

There can be no doubt that, indirectly, the two processes are closely linked when they occur in the same organism. Energy, hydrogen donors, and carbon skeletons required in nitrogen fixation must be derived ultimately from the photosynthetic process and the expansion of the photosynthetic mechanism must in turn depend on nitrogen fixation. Even if there is no specific dependence of the process on hydrogen donors produced photochemically it is possible that these can be utilized, as well as those produced in respiration, for the reduction of elementary nitrogen, just as the reduction of nitrate can evidently proceed photochemically as well as by dark reactions in *Chlorella* (101). The principal carbon compound involved in the entry into metabolism of ammonia, whether it is supplied as such or produced by nitrogen fixation, is presumably α-ketoglutaric acid in both blue-green algae and the photosynthetic bacteria and this could be derived from photosynthetically produced phosphoglyceric acid via the tricarboxylic acid cycle.

The general physiological relations between photosynthesis and nitrogen fixation arising from such biochemical intermeshing have been studied with leguminous plants. The general conclusions of a review of this work by Wilson (114) were that, whereas the total fixation of nitrogen increases, the relative fixation, and hence the nitrogen content of the plant, fall with increasing photosynthesis. At high light intensities there may be a decrease in total nitrogen fixation, evidently because, with an excess of carbohydrate, the nitrogen-fixing system is unable to compete successfully with other synthetic systems for the available combined nitrogen, and thus its expansion is prevented. It may be that the prolongation of the lag period in the growth of *A. cylindrica* with increasing light intensity observed by Fogg (37) arose in a similar way. Using N^{15} as a tracer it has been demonstrated that nodulated plants of *Trifolium pratense* can fix nitrogen in the dark though at a decreasing rate as the period of darkening is increased, 24 hr. predarkening sufficing to stop nitrogen fixation completely (71). The uptake of ammonia is affected by darkening to a lesser extent than nitrogen fixation. In soy bean plants it appears from experiments using C^{14} as a tracer that the product of photosynthesis upon which nitrogen fixation most depends is sucrose (12). Addition of this sugar to slices of excised soy bean nodules markedly increased their fixation of nitrogen as measured by incorporation of N^{15}. These tracer studies also confirmed that α-ketoglutaric acid is an important intermediate for the fixation process. Similar studies on the general physiological relationships between photosynthesis and nitrogen fixation with blue-green algae or photosynthetic bacteria do not appear to have been reported.

Symbioses Involving Nitrogen-Fixing Blue-Green Algae

Blue-green algae, particularly species of *Anabaena* and *Nostoc* which are presumably nitrogen-fixing, frequently occur in association with other plants, e.g., with fungi in many lichens and endophytically in liverworts,

ferns, cycads and angiosperms [for a general review of the older literature on this see (45)]. There is definite evidence that some of the algae concerned can fix nitrogen. Thus Winter (118) obtained the same alga from a number of cycads and showed that nitrogen fixation occurred in bacteria-free cultures. Lhotský (68) has stated that different roots of a cycad, even if from the same plant, may contain different algae and has suggested that this may be a source of confusion in nomenclature. Henriksson (53) isolated in a bacteria-free condition a species of *Nostoc* from the lichen *Collema tenax* and found it to be nitrogen fixing. On the other hand, Lhotský (68) found little, if any, growth of *Nostocs* from *Cycas* and *Anthoceros* and of an *Anabaena* from *Anthoceros* in impure cultures with no supplied combined nitrogen, whereas similar cultures with nitrate showed good growth. However, the media used were somewhat acid, ranging from pH 3.4 to 7.2, and it is possible that nitrogen fixation was not observed because of a specific inhibition by hydrogen ion.

There is some evidence for nitrogen fixation by organisms with associated blue-green algae. Bortels (16) found that *Azolla* with its endophytic *Anabaena* was capable of growth in a medium free from combined nitrogen. A small amount of combined nitrogen was, however, beneficial and allowed of more nitrogen being fixed. Bond & Scott (15) have shown unequivocally, using N^{15} as a tracer, that two lichens, *Collema granosum* and *Leptogium lichenoides*, and the liverwort, *Blasia pusilla*, all of which contain *Nostocs*, are able to assimilate elementary nitrogen. Douin (30, 31) found that nodules of *Cycas circinalis* and *Stangeria paradoxa* containing *Anabaena cycadeae* had higher nitrogen contents than normal roots, a fact which suggests, but does not prove, that nitrogen fixation occurred in the nodules.

The degree of specificity of these symbioses varies. Winter (118) and Douin (30) both found that algae isolated from cycads were capable of heterotrophic growth but were scarcely capable of phototrophic growth. Bortels (16) was unable to grow the *Anabaena* endophytic in *Azolla* apart from its host. In such instances the alga is evidently dependent on its host for carbohydrates and perhaps growth factors. On the other hand, the *Nostoc* from *Collema tenax* (53) was capable of independent growth in inorganic media, evidently requiring no organic growth factors. Harder (52) succeeded in isolating *Nostoc punctiforme* in pure culture from *Gunnera scabra* and found that it was capable of phototrophic growth as well as growth in the dark on a variety of organic substrates. Although he observed growth in the absence of added sources of combined nitrogen, he attributed this to nitrogenous impurities in the agar used rather than to nitrogen fixation. Schaede (89) has studied the cytology of the symbiosis between *Nostoc* and *Gunnera manicata*. The algal trichomes penetrate into the cells of the higher plant and there grow more vigorously than they do outside. However, following such intracellular growth the alga appeared to be incapable of independent existence. The extent to which the nonalgal partner benefits from such associations and the form in which it receives the nitrogen fixed have been scarcely inves-

tigated. Henriksson (53) found the *Collema Nostoc* to excrete into the medium as much as a quarter of the total nitrogen fixed in pure culture. Fogg (39) found the extracellular products of *A. cylindrica* to be largely unavailable as a nitrogen source either to the *Anabaena* itself or *Chlorella*, but it does not follow that the extracellular products of the *Collema Nostoc* are of a similar nature, nor, if they are and are liberated in the intact lichen, that the fungal partner is unable to make use of them. Bond & Scott (15) have put forward reasons for believing that there is transfer of nitrogen between the *Nostoc* endophytic in *Blasia* and its host.

It may be noted that symbiosis between green algae and *Azotobacter* is evidently not important in promoting nitrogen fixation (95).

The Role of Nitrogen Fixation by Photosynthetic Organisms in Natural Economy

Blue-green algae.—Species of *Nostoc*, *Anabaena*, and other blue-green algae likely to be capable of nitrogen fixation are widely distributed and frequently abundant in soils and freshwater in temperate, tropical, and polar regions, but occur to only a minor extent in marine habitats (45). Attempts to assess their importance as nitrogen-fixers have, however, been made only for a few situations.

The quantitative importance of blue-green algae as nitrogen-fixers in temperate soils appears slight (95), although several nitrogen-fixing species have been isolated from such sources (16, 32) and their abundance increases with the fertility of the soil (16). Hérisset (54) has demonstrated considerable fixation of nitrogen in cultures of *Nostoc commune* in which conditions approximated those under which this alga grew during humid weather on a sandy substratum in the Angers arboretum. Odintzova (81) attributed the formation of nitrate deposits in the Western Pamirs to nitrogen fixation by *Gloeocapsa minor* but, although appreciable fixation was observed in cultures of this alga these undoubtedly contained other organisms, and it has not yet been confirmed that any member of the Chroococcales is capable of nitrogen fixation.

In certain tropical soils nitrogen fixation by blue-green algae can undoubtedly make a major contribution to soil fertility. Growth of these organisms in rice fields is often luxuriant, many of the species concerned have been shown to be nitrogen-fixing (27, 92, 111), and various lines of evidence indicate that these are the principal agents responsible for maintaining the nitrogen status of the soil. Singh (93) has described how alkaline "Usar" lands in northern India may be reclaimed by enclosure to form shallow ponds to encourage the growth of blue-green algae in the rainy season. The organic content, nitrogen content, and water-holding capacity of the soil may be improved considerably by this means with the result that successful crops may be grown on it. De & Sulaiman (28) found that both growth and nitrogen fixation by blue-green algae were increased in soil culture in the presence of rice seedlings. This stimulating effect was attributed to the in-

creased supply of carbon dioxide resulting from the respiration and decomposition of the roots of the higher plant. Phosphate and calcium supplies appear to be important limiting factors for the growth of blue-green algae in certain paddy-field soils (28, 82, 83). In paddy soils treated in various ways gains in total Kjeldahl nitrogen have been found to parallel algal growth, both being poor in soils kept in the dark (82). Inoculation of soils with algae is only sometimes effective in increasing their nitrogen content (83). De & Sulaiman reported higher crop yields and increased soil nitrogen content associated with abundant algal growth in pot cultures (29). Such effects were not observed, however, until after three years, and Engle & McMurtrey (34) found that *Nostoc muscorum* was not able to make available sufficient combined nitrogen to improve the growth of tobacco plants in nitrogen-deficient media. These results indicate that no appreciable proportion of the nitrogen fixed by the algae is directly available to the higher plants which can evidently only benefit after decomposition of the algae has occurred. Watanabe *et al.* (112), on the other hand, have reported increased growth of rice plants in various types of culture inoculated with nitrogen-fixing algae. In water cultures with *Tolypothrix tenuis* the lengths of the leaves were greater than those of plants in control cultures by an average of 17 per cent and the number of ears by 30 per cent. It is not clear whether this improvement can be attributed directly to fixation of nitrogen by the alga. Increased yields, apparently within one season, from paddy fields inoculated with *Tolypothrix* are also reported, the nitrogen fixation attributable to this alga amounting to 30 lb. per acre. Another alga, *Anabaenopsis*, known to fix nitrogen in culture, produced no appreciable effect, however.

Heavy "blooms" of blue-green algae are characteristic of many eutrophic fresh-waters in both temperate and tropical regions. An assumed nitrogen-fixing capacity of certain of the species concerned has been considered to play an important role in the nitrogen economy of the bodies of water containing them (1, 57). However, two of the blue-green algae most frequently forming blooms, *Diplocystis* (*Microcystis*) *aeruginosa* and *Aphanizomenon flos-aquae*, have been found not to fix nitrogen (113) and the various planktonic species of *Anabaena* which may also form blooms have not yet been examined critically from this point of view. Sawyer (88) has shown that lake water, fertilized with sewage effluent from which nitrogenous compounds had been removed, supported a vigorous growth of blue-green algae accompanied by a considerable fixation of nitrogen. Removal of phosphates from the effluent was found to be a reliable means of preventing this growth. The water of certain types of hot spring generally contain no nitrate and *Mastigocladus laminosus* growing in them has been found to be also capable of growth in impure laboratory cultures in media free from combined nitrogen at a temperature of 48°C. (107). Proof that this species is able to fix nitrogen (38) makes it likely that it plays an important part in the nitrogen economy of the hot springs in which it occurs.

Analyses by Williams & Burris (113), Fowden (42) and Magee & Burris

(74) show that, as far as the amino acid composition of their bulk proteins is concerned, nitrogen-fixing blue-green algae do not differ markedly from other plants and should be adequate for animal nutrition.

Photosynthetic bacteria.—Scarcely any information appears to have been published that helps in the assessment of the importance in nature of nitrogen fixation by these organisms. Photosynthetic bacteria are widely distributed in marine and freshwater habitats and in certain situations are abundant (see 67, 121). The formation of considerable sulphur deposits in Cyrenaican lakes, for example, has been attributed to the activity of *Chromatium* and *Chlorobium* in conjunction with sulphate-reducing bacteria (25). However, the water in which this impressive development was attained had about 5 p.p.m. of potassium nitrate and it is unlikely that nitrogen fixation would occur in the presence of such a concentration of available nitrogen. It may be surmised that most environments in which photosynthetic bacteria flourish contain similar fixation-inhibiting concentrations of combined nitrogen. However, this does not preclude the possibility that in early geological eras their nitrogen-fixing activities may have been important.

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ENDOGENOUS RHYTHMS IN PLANTS[1]

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Introduction with Definitions

Endogenous rhythm is the term used in describing biological processes which alter periodically although external conditions remain constant. The movement of a pendulum is analogous to a biological endogenous rhythm. As in pendular movements, biological systems also require an impulse to set them in motion. A single impulse brought about by some external factor such as a transition from darkness to light or from a low to a high temperature may evoke an endogenous rhythm which was previously unrecognizable. Very often these external factors are also decisive as time determinants, fixing the position of maxima and minima of the rhythm which occur following the single impulse while the external conditions are constant. The time required for one period of the endogenous rhythm, i.e., the duration of a single period, however, may depend upon external factors such as temperature.

An endogenous rhythm must not necessarily be considered of hereditary origin, although it often may be. It is in some cases obviously induced by some preceding external rhythm as, for instance, by light-dark periodicity. In such cases a single impulse, such as transition from light to darkness or darkness to light is not sufficient to make the rhythm manifest.

An endogenous rhythm usually can be followed under constant external conditions only for a relatively short time. Sometimes only two to three periods can be registered if no further impulses from some external stimulus follow.

Rhythms With Short Periods

In flagellar movement obviously an endogenous rhythm with a very short period is involved. We cannot as yet conceive the mechanism of this rhythm. Recently we have gained some knowledge as regards the mechanism of the movement itself, and we may assume that it depends on contractions in the individual fibrils of the flagella. However, we do not know why these single fibrils contract so regularly and in definite orderly sequence. It appears at any rate that rhythmic contractions of the individual protein molecules are of importance. This phenomenon also occurs in several other protoplasmatic processes. It is probably involved in the pressure fluctuations studied by Kamiya & Abe (65) in the streaming protoplasm of *Physarum*, in which a complete period consisting of backward and forward movement lasts approximately 90 sec. at room temperature and 5 min. at 10°C. This

[1] The survey of literature pertaining to this review was concluded in September, 1955.

periodicity again might be related to the rhythmic torsional oscillations which occur with a similar period in freely suspended strains of *Physarum* plasmodium (67). Obviously the property possessed by protein chains to contract and relax themselves periodically is widely distributed. Kamiya & Nakajima (66) found that "the rhythmic functioning of protoplasm . . . has been confirmed to be operative no matter whether the protoplasma is in a state of sol or that of gel." They therefore argue that this rhythm may be considered to be a "more deep-seated attribute of protoplasm."

We are confronted with similar phenomena in the case of higher plants as regards endogenous rhythms occurring inside the tissues. We may mention, for example, the nutational movements of higher plants. These movements manifest themselves in the mode of growth, especially of the apical parts of shoots. Instead of growing straight upward, the shoots perform

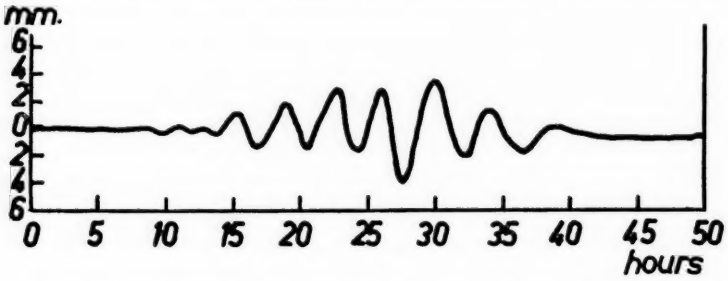


FIG. 1. Nutations of the coleoptile of *Triticum*. Ordinate: Declination of coleoptile tip from zero position [after Arnal (1)]

pendulous or revolving movements. In winding shoots or tendrils these movements are particularly regular. They are brought about by unequal growth on different sides of the stem. The line of maximal growth rate migrates around the stem, describing a complete circle (or a more or less irregular line), usually within a few hours and sometimes even more rapidly. Thus, there appear to be endogenous pulsations of growth rate on each individual flank of the shoot or of the tendril. With coleoptiles of *Triticum*, Arnal (1) observed nutational movements reaching amplitudes of 5 to 8 mm., the length of one period in this case being approximately 3 hr. (Fig. 1). Arnal relates this rhythm to a corresponding fluctuation of auxin supply, a hypothesis that still requires experimental proof. Moreover, it leads one to the question how such fluctuations of auxin output can originate.

Further efforts in the direction of achieving a deeper insight into the course and mechanism of these nutational movements have been made by Tronchet *et al.* (93) and by Baillaud (5). The Q_{10} value for the speed of rotation in *Cuscuta odorata* for temperatures between 10° and 25°C. is about two (5).

All observations and findings on endogenous rhythms of protoplasmatic properties are of particular value in explaining these growth rhythms. Rhythmic fluctuations in viscosity have been observed by Stålfelt (90) and Virgin (95, 96) in *Helodea*, *Spirogyra*, and *Mnium* which were cultivated un-

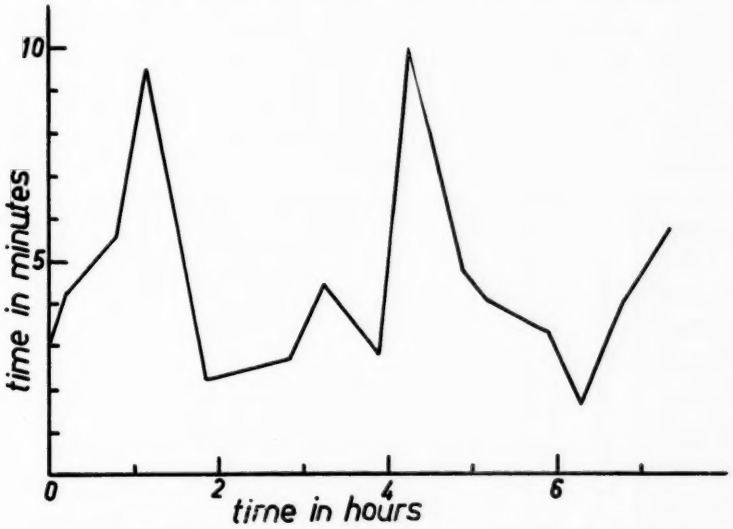


FIG. 2. Fluctuations of viscosity in leaf cells of *Helodea densa*. Ordinate: Viscosity (time required for complete translocation of chloroplasts by centrifuging) Abscissa: time in hours [after Stålfelt (90)].

der constant conditions. Phases of equal viscosity represented by minima and maxima lay 2 to 3 hr. apart (Fig. 2). In constant darkness the rhythm was found to disappear; however, it could be induced anew by placing the cultures in light.

ENDOGENOUS DIURNAL RHYTHMS

Examples and regulating factors.—The first observations of the existence of an endogenous diurnal rhythm in plants were made very long ago. Investigations on diurnal leaf movements initiated the idea that an endogenous component was involved. More than 50 years ago the first reports on endogenous diurnal variations of growth and other physiological processes were reported. Numerous publications on endogenous diurnal rhythms have followed during the last 25 years. The rhythms proved to be not only endogenous but also hereditary, i.e., not induced at early stages of the individual development. The rules of regulation through light-dark cycles and other factors were studied [cf. list of literature in Bünning (19) and Flügel (43)].

The work of Bühnemann (13 to 16) on rhythmic sporulation in *Oedogonium cardiacum* can be classified under recent investigations on the participation of an endogenous diurnal rhythm in individual physiological processes. Discharge and germination of spores in *Oedogonium* are regulated to a large extent by light-dark cycles, as is the case with nearly all the endogenous diurnal processes. The participation of an endogenous component can be observed only when the continuation of the rhythm is investigated under constant external conditions. This continuation can be observed for at least a few days and, in several experiments, even after 64 days of permanent darkness. Thus, there is an endogenous diurnal rhythm of a sporogenous tendency. The role of the light-dark alternations lies, not in inducing this

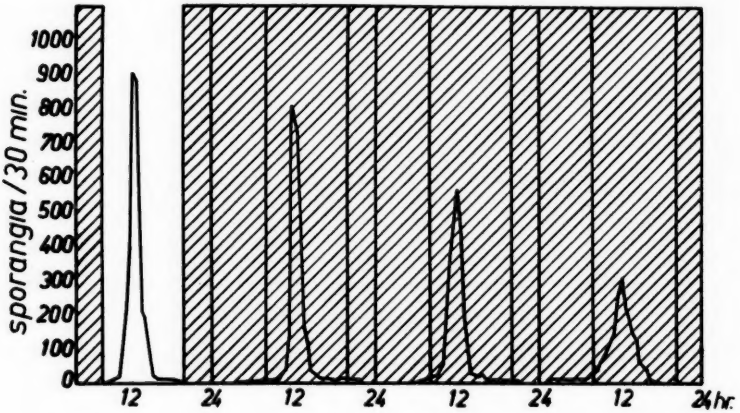


FIG. 3. Frequency of sporangia-projection in *Pilobolus sphaerosprous* in 12:12 hr. light-dark alternation with following continuous darkness. Dark times hatches [after Uebelmesser (94)].

rhythm, but in determining the times of its maxima and minima. And so the length of a single period within this rhythm cannot be modified by a preceding abnormal light-dark periodicity. Thus, an adaptation to light-dark cycles of 9:9 or 15:15 hr. is impossible. Following such cycles, the 24 hr. endogenous rhythm reappears. This observation agrees with the data established for higher plants (68). The observations of Schmidle (82) and Uebelmesser (94) on the ripening of *Pilobolus* sporangia can well be compared with the investigations on *Oedogonium* (Fig. 3). In this case, too, the participation of an endogenous diurnal rhythm has been clearly stated. The same is true in spore discharge of the pyrenomycete *Daldinia concentrica* according to the investigations of Ingold & Cox (60). In continuous darkness periodic spore discharge was maintained for 12 days. In continuous light it ceased after 2 to 3 days. Periodicity was at once re-established as soon as the plants were

returned to alternating light and darkness. In *Daldinia* it is possible to induce a new periodicity following cycles of 6 hr. light plus 6 hr. darkness. This is not contradictory to the participation of an endogenous diurnal rhythm. The experimental results point directly to a superimposing of two 24 hr. rhythms. In permanent darkness the 24 hr. rhythm is re-stablished. Similar facts are known for higher plants.

Recent investigations by Jones (62) on *Callitriche* and especially the work of Ball & Dyke (6) on *Avena* seedlings demonstrate the participation of an endogenous rhythm in diurnal growth variations. After the single impulse caused by transferring the seedlings from continuous red light to continuous darkness, an endogenous diurnal rhythm of growth is established in the following permanent darkness, which lasts for 2 to 3 days. This fact indicates that a preceding diurnal light-dark alternation is not necessary for the manifestation of this endogenous and hereditary diurnal rhythm. The first peak of growth is registered 16 hr. after the impulse, the second being after 16 plus 24 hr. and the third after 16 plus 48 hr.

The nyctinastic movements of leaves have been made the object of detailed investigations to determine the functions of light and darkness as the time determinants of the endogenous diurnal rhythm. Classical investigations of Pfeffer made more than half a century ago stated the probable involvement of a diurnal rhythm, and that the transition from darkness to light had a marked influence on the position of maxima and minima. This is true for *Phaseolus* and certain other leguminous plants. [For list of literature see Bünning (19) and Flügel (43)]. In other cases, e.g., *Xanthium* (18), the transition from light to darkness is more effective. Regulation through different light-dark cycles, light intensities, etc. has been recently studied by Flügel (43) and by Bünsow (29, 30, 31). According to Flügel, the maximal declination in the primary leaves of *Phaseolus multiflorus* is reached approximately 17 hr. after the beginning of the light phase, the duration of the light phase being rather unimportant. That regulation through abnormal light-dark cycles is limited in a similar fashion was found by Kleinhoonte (68) in her earlier investigations on *Canavalia*. If the length of one light-dark cycle is less than 18 hr. (in place of the normal 24 hr. cycle), the endogenous component becomes so markedly apparent, that the rhythm can no longer adjust itself to the duration of the light-dark cycle.

We may state, in summary, that light-dark cycles can shift the maxima and minima of the rhythm to any time of day, but the length of the single periods can be regulated to a limited extent only. If the abnormal light-dark cycles are replaced by continuous darkness, the regulation of the length of the periods immediately disappears, and the 24 hr. rhythm is re-established. This corresponds to what we have previously seen in the case of *Oedogonium*.

Bünsow's investigations (29, 30, 31) refer to the diurnal movements of petals of *Kalanchoe blossfeldiana*. Rules concerning light and dark regulation are similar to those for other leaves. In tissue cultures, too, endogenous fluctuations in growth and turgor pressure have been established (39).There

is no need to emphasize in all these cases, that the rhythm continues for some days (usually not more than a week) even with the complete cessation of light-dark alternation.

Although several authors have studied the regulation of the endogenous diurnal rhythm through light and darkness, little is known of the decisive photoreceptor. According to Bühnemann (15) only light between 400 and 500 mμ is effective, the maximal efficiency being reached between 400 and 450 mμ. This points to riboflavin as the decisive receptor in *Oedogonium*.

Regulation through temperature cycles.—The endogenous diurnal rhythm can also be regulated by cycles of high and low temperature. It is known that this influence, in the case of higher plants, is of a lower order than that of light-dark cycles. This is valid also for *Oedogonium* (14). In all these cases, one comes to the conclusion that cycles of high and low temperature regulate the rhythm in such a way that the physiological phase, which normally falls during the dark period, now occurs during the period of low temperature and the phase which normally falls during the light period, now runs during the period of high temperature (14). Like light-dark cycles, temperature cycles have a regulative effect only when the length of one cycle (i.e., one period of low plus one period of high temperature) does not deviate too much from 24 hr. In *Oedogonium* the endogenous rhythm may follow temperature cycles of 12:12 hr. and of 9:9 hr. But, when the temperature cycles deviate further from 24 hr. periods, the same thing happens as when deviation in light-dark cycles is too great (68), i.e., the endogenous rhythm goes its own way with 22 to 26 hr. periods (14).

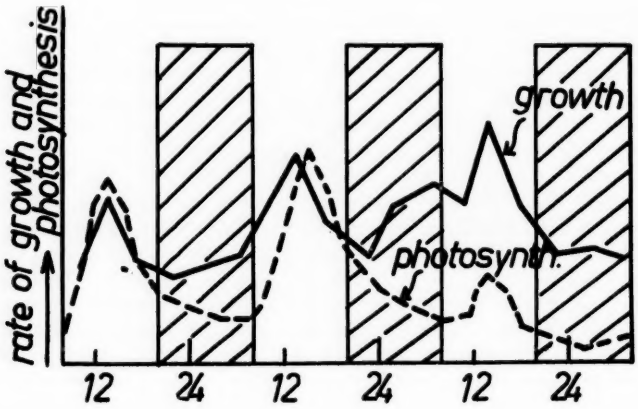
Deviations from exact 24 hr. rhythm.—Very often during permanent light or permanent darkness the length of one period differs noticeably from that of 24 hr. Thus, for example, cycles of 26 hr. duration can often be measured. In *Oedogonium* at 25°C. Bühnemann (13) found cycles of about 22 hr. Also in *Oedogonium* the length of the periods is regulated to a certain extent by the light-dark alternation. Therefore, we can recognize the actual cycle length of the endogenous component of the rhythm only under constant conditions. Thus the really endogenous component of the rhythm may show a tendency towards cycles which deviate somewhat from the 24 hr. period. About 25 years ago the existence of an endodiurnal system was indicated by this deviation of the length of one period from the exact duration of the external diurnal rhythm (17). As a result of the regulative effect of light-dark alternation or temperature cycles, we can find these derivations only in permanent light or permanent darkness. But under these conditions we may, within a greater population, even find hereditary differences in the cycle length (18).

Temperature and length of cycles.—As early as 25 years ago (17) it was shown that the length of the periods measured under constant external conditions is only a little shorter at high temperature than it is at low temperature. The Q_{10} values have been found to be approximately 1.2 in *Phaseolus*. Even more remarkable are the results with the previously mentioned growth

rhythms in *Avena* coleoptiles. Between 16° and 28°C. there is little or no influence of temperature on the length of the periods (6). As already mentioned, at 25°C. *Oedogonium* shows cycles of 22 hr. (13). At 27.5° cycles increase to 25 hr.; at 17.5° they decrease to 20 hr. (14). Here, the speed of the rhythm decreases at higher temperatures, the Q_{10} being only 0.8. From these facts we may conclude that the endogenous rhythms result from the interaction of several processes, each possessing a different Q_{10} value.

Metabolic basis.—Further insight into the mechanism of the endogenous rhythm becomes possible only by investigating metabolism. In this connection the recent observations on the participation of an endogenous component in the diurnal drift of guttation and bleeding are of importance (36, 40, 48, 49, 55). Obviously such fluctuations in excretory functions are related to metabolic processes. Also the recent demonstration by Busch (32) of changes in plastid shape in *Selaginella serpens* brought about through an endogenous diurnal rhythm can be considered as a key to the problem. The changed status of plastids perhaps is connected with variations in their enzymatic capacities. Light-dark cycles regulate the changes in plastid shape which, however, continue for two to three days either in continuous light or in continuous darkness. Obviously these changes are related to the already known endogenous diurnal variations in acidity which in turn points to a rhythm in metabolism (20, 21). Furthermore, it is important to study the fluctuations of enzymatic activity itself. The first observations in this field were made about 15 years ago. Meanwhile, our knowledge on this subject has increased (52). Ehrenberg (37, 38) studied the variations of phosphatase and amylase activity in *Phaseolus multiflorus*. Rhythmic change in phosphatase activity in *Kalanchoe blossfeldiana* was also investigated. In particular the regulative action of light-dark cycles has been investigated. In *Phaseolus* the enzyme activity maximum appears about 9 to 12 hr. after the beginning of a light period. In experiments with *Spinacia*, Venter (94a) found a continuation of the rhythm in amylase activity even after five days of continuous light.

It is of particular interest to note that the endogenous diurnal rhythm is connected, not only with these quantitative variations in the enzyme activity, but also with qualitative changes in the whole character of metabolism. Evidently during each of two 12 hr. phases, certain enzymes become dominant which are less active during the alternate phase. Former investigations have already shown that in one phase there are catabolic and in the other phase anabolic processes predominating (20, 21). This has been proved again by recent studies of Pirson *et al.* (74, 75, 76), Neeb (72), and Schön (84), who worked on *Hydrodictyon*. A periodicity of growth, photosynthesis, and respiration in the dark has been observed (Fig. 4,5). Under constant conditions (such as continuous light) following a 12:12 hr. light-dark periodicity, these periodic variations continue. These continued rhythms should be designated as endogenous fluctuations because the fluctuations actually can be observed while the external conditions are constant. Also the regulation



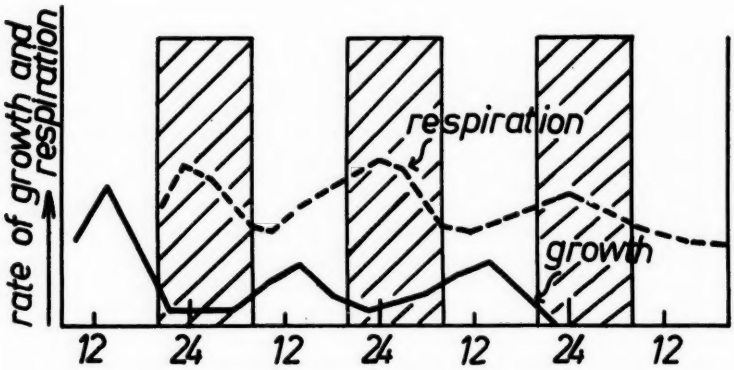


FIG. 4. (above) Growth rate and rate of photosynthesis in *Hydrodictyon* in continuous light after preceding light-dark cycles of 12:12 hr. Times of previous darkness are hatched. Abscissa: daytime [after Pirson, Schön & Döring (74)].

FIG. 5. (below) Growth rate and respiratory O_2-uptake of *Hydrodictyon* in continuous darkness. Times of previous darkness are hatched. Abscissa: daytime [after Pirson, Schön & Döring (74)].

of this metabolic rhythm through light-dark cycles is quite similar to the previously mentioned diurnal phenomenon. However, in *Hydrodictyon* a special situation arises in that the length of the physiological period, occurring under constant conditions, depends upon the length of the cyle of the

preceding light-dark period. Thus, one can induce rhythms with either 12 or 18 hr. cycles in place of 24 hr. cycles, through a light-dark change in which one cycle (one light plus one dark phase) is of 12 or 18 hr. duration respectively. This difference in behavior is remarkable, when one compares it to the previously discussed rhythms. Nevertheless, these induced rhythms in *Hydrodictyon* may be supposed to represent a modification of the endogenous diurnal rhythm. The only difference lies in the fact that in *Hydrodictyon* under constant conditions the periodicity continues following the modified lengths of the single period. In the cases described before, this modification is also to be found but it does not continue in permanent darkness or permanent light. Rather, under constant conditions, the periodicity always shows periods of approximately 24 hr. In any case, during these two different phases in *Hydrodictyon*, the character of the metabolism corresponds very well with the previously discussed diurnal rhythms of other plants, i.e., alternating periods of fast and slow growth, or periods of high photosynthetic efficiency alternating with those of intensive respiratory efficiency.

Diurnal variations of metabolic activity such as CO_2 production have several times been described in the case of higher plants (20). Recent investigations with *Kalanchoe blossfeldiana* were published by Schmitz (83). The CO_2 evolution shows an endogenous rhythm, in which a minimum is reached 12 hr. following a long dark period and a maximum 12 hr. later. However, this rhythm fades away very quickly. In this connection, mention should be made of the investigations of Gregory, Spear & Thimann (47), who worked with the same plant. It is interesting to note that CO_2 fixation in the dark and CO_2 evolution in the light increase up to a maximum and then decrease. It would be worthwhile discovering whether these changes have anything to do with endogenous diurnal rhythm. Actually, according to Nuernbergk (72a), in several species of *Bryophyllum*, *Aloe*, *Gasteria*, and *Kalanchoe* these rhythms are maintained for several weeks in continuous light.

Certain internal layer formations in plants point to qualitative diurnal variations in enzyme activity. This is particularly true of the layers in starch grains. It has been shown by Roberts & Proctor (80), as well as by Mes & Menge (71), that this formation of layers is continued even under constant external conditions. Moreover, Bünning & Hess (27, 56) have proved that the formation of about one double layer occurs within 24 hr. This leads us to relate it with endogenous diurnal rhythm.

To elucidate the mechanism of endogenous diurnal rhythms Galston & Dalberg (45) offer the explanation that the endogenous diurnal rhythm begins with the adaptive formation of indoleacetic acid oxidase, which is attributable to a gradual increase in the auxin level. Consequently after adequate intense formation of enzyme has taken place, auxin inactivation sets in and the result is a lower auxin level. Diurnal fluctuations in auxin concentration are connected with the endogenous diurnal rhythm (7). For example, *Kalanchoe blossfeldiana* leaves and petals show a maximum of auxin production about 3 to 6 hr. after the beginning of a light period. In continu-

ous darkness, followed by light-dark cycles, this maximum is reached 6 plus 24 hr., 6 plus 48 hr., etc. after the beginning of the last light period.

Rau (79) studied the effects of light periods of short duration on dry matter production and nitrogen uptake in seedlings (*Perilla, Amaranthus, Lactuca, Brassica, Plantago, Hyoscyamus*). These effects depend on the time of day when the plants were exposed to short light periods. Cycles of 24 or 48 hr. have been used, the main light period being 6 or 12 hr. respectively. The influence of an additional 3 hr. light period (interrupting the 18 or 36 hr. dark period) on dry matter production and nitrogen content has been studied. The additional light had a maximal effect if presented 6 or 6 plus 24 (i.e., 30) hr. after the beginning of the main light period. Thus, there were two maxima of light influence, which were approximately 24 hr. apart. The smallest effects were attributable to additional light that had been given 18 or 33 hr. after the beginning of the main light period. Therefore these phases, which start endogenously and then fade away, are about 24 hr. apart. In similar experiments Clauss (34) observed that under the influence of these same periods of illumination, formation of plastid pigments also depends on the time of day when the additional light is offered. Here again the maximal synthetic efficiency in 48 hr. cycles is reached at 6 or at 6 plus 24 hr. after the beginning of the main light period.

Great caution is necessary in interpreting these observed fluctuations in enzyme activities and metabolism. Probably all these variations are the result of endogenous rhythms and not their causes. The above mentioned endogenous alternations of metabolic activities observed in *Hydrodictyon* cannot be explained simply as a mutual correlation of the well known metabolic processes. The rhythm is neither enhanced nor retarded by changing the CO_2 or the glucose concentration suggesting that no simple mechanism is involved (84).

Efforts have been made to explain the mechanism of physiological self-regulation in endogenous diurnal rhythms from the standpoint of energy balance (78, 88). It would be premature to construct hypotheses at this stage. Nevertheless, it may be important to note that a phase of energy accumulation alternates regularly with a phase of energy turnover. Perhaps this takes place in all living cells and thus a diurnal rhythm is maintained (30). This seems to be a fundamental principle for the functioning of all living cells.

According to Bühnemann's experiments with *Oedogonium* (16), the endodiurnal system can not be disturbed by poisons like NaCN, arsenate, 2,4-dinitrophenol, NaF, and others. Therefore, we cannot connect the mechanism of the rhythm with the activity of enzymes which are inactivated by these substances. Especially the cytochrome system cannot be involved. In all cases, the periodicity of the physiological processes remained clear as long as the poison did not fully suppress the physiological processes by damaging or killing the cells. On the other hand, there was also no influence of adenosine triphosphate, riboflavin, sucrose, or indoleacetic acid.

Tamiya (91) discusses a diurnal phenomenon in his *Chlorella* experiments. The algae were subjected to a 12:12 light-dark rhythm. In this case, results showed an antagonism of cellular efficiency which moreover finds its expression in different cell shapes. This antagonism may be interpreted as being one between photosynthesis and formative metabolism (91).

The formation of autospores is also associated with the phase of formative metabolism. This scheme fits closely the described facts on endogenous rhythm. It fits in the mentioned rhythms of enzyme activity, in the alternations of assimilation and dissimilation, and also in the described rhythms of sporulation.

Finally, the diurnal rhythm of mitosis can also be mentioned in this connection. Bühnemann (15) was able to show that this rhythm is governed by the endodiurnal system too.

Relation to photoperiodism.—Although the mechanism of the endogenous rhythm is not yet fully understood, it has become clear from the available evidence that two phases, each of about 12 hr., alternate during the course of the endogenous diurnal rhythm. These phases differ in a quantitative manner with respect to certain partial processes and to certain others in a qualitative manner. And so it is nearly self-evident that during these phases, plants respond to external stimuli quantitatively or even qualitatively with different reactions. Primarily, they react differently to light and darkness. For normal development, a light-dark rhythm must be presented and this rhythm should be in the period of the endogenous rhythm, i.e., in a 24 hr. cycle.

If light cycles are chosen which are too fast or too slow, considerable developmental disorder may appear. Highkin & Hanson (57) studied such a preference of cycles of 24 hr. duration, particularly in tomatoes, and they associate the above results with the endogenous diurnal rhythm. In *Hydrodictyon* in continuous light, marked developmental disorders appeared which led to the formation of pathological forms (72). Members of the Desmidiaceae also require a regular light-dark change for their normal development. Continuous light, even of relatively low intensity, produces very harmful effects (63).

The necessity of dark periods reminds us of the photoperiodic reaction. According to the reviewer's conception, the essential nature of all photoperiodic reactions consists in the fact that the two phases of the endogenous diurnal rhythm respond to light, with different reactions, often even with antagonistic ones. This conception of the mechanism of photoperiodic reactions does not differ fundamentally from the conception developed by other authors. Usually photoperiodism is explained by postulating that light and darkness respectively induce certain reactions. Very soon this postulation had to be supplemented with the additional assumption that these induced reactions require a definite length of time. Moreover, it can be concluded that these reactions must be followed by other reactions which in turn are characterized by a definite time requirement. This reviewer is of the opinion that

these interpretations should be fitted into our existing knowledge of endogenous diurnal rhythm with the conception that the induced reactions are phases of the endogenous rhythm regulated by the light-dark alternation. For example, it is possible to distinguish in short day plants (such as Biloxi soybeans) a high intensity light reaction, a dark reaction and a low intensity light reaction (69). The high intensity light reaction may consist of photosynthetic processes. With its characteristic features, it corresponds quite well to that phase of the endogenous rhythm which is characterized by high photosynthetic efficiencies and which is also regulated by a light period as is the high intensity light reaction itself. The dark-reaction, as is well known, starts several hours after the high-intensity light reaction and has features which are comparable to the other phase of the endogenous diurnal rhythm. Therefore, one is nearly compelled to assume that the several reactions in the photoperiodic responses are nothing but phases of the endogenous diurnal rhythm.

This view has been emphasized for the last 20 years but hitherto has been accepted only to a small extent. This may be explained by the fact that endogenous diurnal rhythms are not known to many plant physiologists. Moreover, frequently in critical discussions no notice is taken of the fact that the endogenous diurnal rhythm can easily be regulated by the light-dark alternation. Because of the ease of their regulation, one gets the impression that these are induced processes, whereas in actuality these processes are regulated phases of an endogenous rhythm. The dependence on external factors is indeed more marked than it seemed before. In the earlier investigations on endogenous rhythm, etiolated seedlings were used, which were kept under constant conditions from the beginning of germination. In such plants, the endogenous rhythm was more evident. Here, in particular, the rhythmic processes following an impulse produced by a light stimulus, continue much longer in uninterrupted darkness (generally for a week), whereas in plants cultivated under normal light conditions, the rhythm may fade within one to three days. Therefore one gets the impression that in normal plants a single process is induced. For that very reason, it is quite natural to speak of an induced high-intensity light reaction which should last for a definite time and must be followed by a dark reaction with a definite length of time.

We have mentioned that in several species of plants the transition from light to dark regulates the endogenous diurnal rhythm much more intensely than the transition from dark to light (23). And so here for a photoperiodic response the induction of a dark-reaction seems to be important (70, 98). That this dark reaction is nothing but the regulation of an endogenous rhythm can be realized from the study made by Lockhart & Hamner (70), who state that the dark-reaction is followed normally be a high-intensity light reaction of approximately half a day's duration.

This second high-intensity light reaction is characterized by a Q_{10} of about one. This lack of temperature dependence also points to the identity

of the induced reaction with a phase of the endogenous diurnal rhythm, where temperature dependence also is very small, as we have already seen.

On the basis of such an assumption, the differences in the intensity of inhibition caused by light breaks at different times during a dark period in the case of short-day plants are easily understood. Similar differences in the intensity of promotion produced by such light breaks at different times during a dark period in the case of long-day plants, are likewise comprehensible. The investigated endogenous changes in the physiological status of the

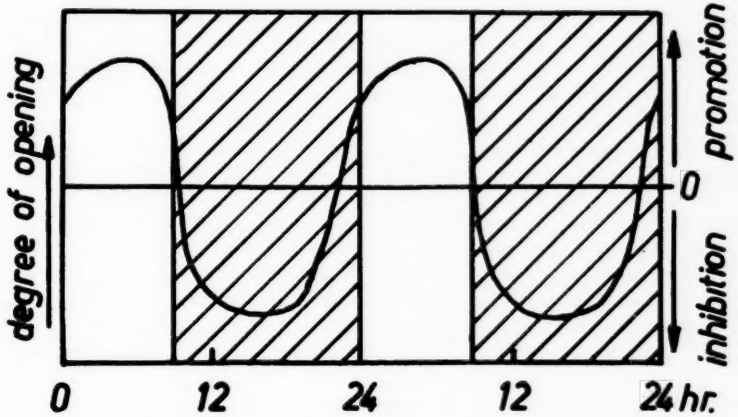


FIG. 6. Relation between opening and closing movements of flowers and dirunally rhythmic changes of photoperiodic light sensitivity in *Kalanchoe blossfeldiana* in light-dark cycles of 9:15 hr. Left ordinate: relative values of aperture of flowers, Right ordinate: Mode of action on flower initiation. Abscissa: hours [after Bünsow (31)].

cells are correlated with their mode of reaction towards light or darkness during the corresponding phases of endogenous rhythm; i.e., each phase of the endogenous rhythm can be associated with a light sensitivity of a definite type and intensity. Thus, in short-day plants, one can predict from the momentary conditions of the endogenous rhythm, whether a light stimulus offered at a given moment would promote or inhibit flower formation.

In several plants, that momentary condition, i.e., the status of the endogenous rhythm, can be judged by registering the movements of leaves and petals. These movements indicate very well the respective physiological condition of the plant and one can predict from the angle formed between the leaf axis and the shoot axis at a certain moment, how that plant would react to light and darkness at that particular moment (Fig. 6) (23, 30, 31).

We have no knowledge of the factors responsible for the different light sensibilities of the two phases in the endogenous diurnal rhythm. This rela-

tion between photoperiodism and endogenous rhythm cannot be discussed in detail in this review. For further discussions see Bünning (23, 24) and Bünsow (29, 30, 31), together with the critical remarks made by Hussey (59), Wareing (98), and Schwabe (85).

Mention should be made at this stage of the recent discovery of the photoperiodic character of a light influence on seed germination (8, 61) which can be related to the endogenous diurnal processes in germinating seeds. The photoperiodic character of this response, is connected with endogenous diurnal fluctuations in the mode of light sensitivity. These are in turn connected with endogenous variations in the metabolism of the seeds (26). Results published by Hagen, Borthwick & Hendricks (50) on sudden changes appearing in the respiration of such seeds, together with those of Borthwick and co-workers (9) on corresponding variation in light sensitivity, are interesting in this connection.

Relation to thermoperiodism.—Apparently the endogenous diurnal rhythm can also be regarded as a cause for the diurnal variation of temperature sensitivity, such as has been demonstrated in seedlings by Schwemmle (86). At different times of the day, the plants were presented with periods of extremely low (9°C.) or high (24°C.) temperatures. The results showed pronounced variation in temperature sensitivity. One may also be led to assume that thermoperiodism, as investigated by Went *et al.* (99), has an endogenous diurnal rhythm as its decisive basis.

A similar relation may also exist in the favorable action of diurnally changing temperatures on seed germination. Here, too, it is obvious that temperature effects depend solely on the moment when high or low temperature acts on the seed. Also, temperature sensitivity in germinating seeds shows periodical changes, and phases with equal sensitivity follow each other at approximately 24 hr. intervals (26).

Endodiurnal systems in animals.—The fact that phenomena of endogenous diurnal rhythms exist in animals too increases our interest in these rhythms. Incidentally, analogous regulability through light-dark cycles can be established (2, 3, 11, 12, 33, 35, 44, 51, 53, 64, 73, 89, 92).

In both plants and animals, regulation by external factors such as light-dark cycles and the continuation in constant external conditions mark the common features of the endogenous diurnal rhythm. Further conformity lies in the fact that in both cases, the influence of temperature on the length of the single periods is very small. A Q_{10} of approximately 1.0 is demonstrable in continuous light or continuous darkness (11). In animals, it can also be shown that the endogenous diurnal rhythm, proceeding under constant external conditions, is not always exactly a 24 hr. rhythm, as periods between 22 and 26 hr. can occur. The far reaching analogies observed in plants and animals make it possible to suppose that there is a common physiological mechanism of the endogenous diurnal rhythm. In certain cases with animals, it has been proved that the endogenous diurnal rhythm is not a continuation of a rhythm induced by external factors before the phenomenon was regis-

tered, but that is a genetically acquired one (3). This again is in harmony with facts established for plants. In animals the endogenous diurnal rhythm is often called "internal sense of time" or "internal watch." It has been found to be very important for animal behavior (cf. 58, 73).

Tidal rhythms.—Tidal rhythms probably are closely related to endogenous diurnal rhythms and take place in several marine animals and plants. Here, length of a single period is approximately 12 hr. (10). Endogenous tidal rhythms have been studied by Fauré-Fremiet in *Chromulina* and *Hantzschia* (41, 42). This 12 hr. rhythm can easily be interpreted as the result of splitting a 24 hr. rhythm. In higher plants too, certain external factors can bring about this splitting. For example, it is possible to superimpose additional oscillations on diurnal leaf movement with the help of certain light stimuli. These oscillations are interposed between normal light-dark cycles and continually reappear at the same time of day.

The investigations of Brown, Fingerman *et al.* (11) made on the crab *Uca pugnax* show clearly the close relation between the endogenous tidal rhythm of a cell and its endogenous diurnal rhythm. Every displacement of the endogenous tidal rhythm caused by a special light condition means also a displacement of the endogenous diurnal rhythm.

In *Euglena*, the relation existing between the two rhythms can be particularly emphasized. In marine forms of *Euglena*, on the one hand, there exist endogenous variations in phototactic sensitivity which enable their cells to ascend and descend with the tides. On the other hand, in other forms of *Euglena*, an endogenous diurnal rhythm of phototactic behavior has been found (77).

Endogenous Rhythms with Longer Periods

Ashby & Wangermann (4) found in *Lemna* endogenous cycles of ageing and rejuvenescence. At 20°C. one complete period requires 73 days, and at 30°C. only 40 days. In this case, therefore, the endogenous rhythms, in contrast to endogenous diurnal rhythms, are markedly dependent on temperature. Ashby & Wangermann have tried to relate these variations to fluctuations in the content of growth promoting substances, but experiments have shown that the regulating factors are neither auxins nor adenine. (97)

In marine animals and plants, lunar periodicities are widely distributed, e.g., periodicities in gamete production (87).

Endogenous Annual Rhythm

It has been known for a long time that dormancy and activity in plants can change even under constant external conditions. Thus, in autumn, the commencement of dormancy in the buds of many plants begins in spite of unchanged external conditions. The same can be said of reactivation in spring. Seeds, too, behave in a similar way. However, in many cases, even under constant conditions for several years, there are changes in activity (either an increase or a decrease) and periodical variations may occur as well.

This has been established by long term observations on trees in uniformly humid tropical regions. In the laboratory it becomes more unequivocal. As an example, the experiments by Henssen (54) on *Spirodela polyrhiza* can be mentioned. Plants were kept at 20°C. in continuous light, and at definite intervals the number of winter shoots formed in several experimental sets were tested under suitable conditions. Over the period from November until February, the number of winter shoots was approximately 100. By May the number dropped to zero, then increased over July and August to a value greater than 100. Here the value remained until the following March when it dropped abruptly to zero once more.

Such a phenomenon could also be demonstrated in tissue and organ cultures (cf., 46). These alterations in the degree of dormancy are correlated with metabolic and protoplasmatic changes [cf., Pirson & Göllner (76)]. At least many of these changes (e.g., enzyme activity, respiration rate, and permeability) are evidently just the result of the changes in activity or can be considered as accompanying phenomena, but not their cause. The lack of simple explanations becomes particularly clear when we consider stored air-dried seeds which show variations in germination rate of samples tested at different times. In some of these cases a definite relation to an annual periodicity is revealed. Remarks in this connection are to be found in the older literature. More recent investigations have been reported by Bünning & Bauer (25), Bünning *et al.* (22, 28), and Ruge & Liedtke (81). Here it is particularly interesting to note that the velocity of the course taken by the rhythm is clearly independent of temperature. Storage at 55°C. and at −22°C. did not result in significant differences in the length of a single period which was always about 12 months. Moreover, extreme dessication, storage in nitrogen, oxygen, or CO_2, etc., did not markedly influence the course of the rhythm. Therefore, it may be assumed that physico-chemical processes, e.g., variations in the colloidal status, in the dormant seeds should be held responsible for the changes in germination capability and not chemical processes. The only indication as to the nature of these changes exists in the fact that the capacity to bind water may change (25, 81).

Presumably all the other changes which can be observed in dormant seeds, for example, changes in enzyme activity, in growth substances, etc., should be considered the result of these unknown primary processes. Experimentation would be necessary to determine whether changes in the activity of buds and other dormant organs are based on a similar phenomenon. Perhaps here, too, the changes in the content of enzymes, growth substances, inhibitors, etc., which have long been studied, should be considered as secondary results only.

Summary and Conclusions

In the plant kingdom, endogenous rhythms possessing periods of quite different duration are widely distributed. Some of these rhythms do not show any similarity whatsoever to the external rhythms. Others do show an

evident similarity in that the length of their periods corresponds to that of some external (e.g., annual, diurnal, or tidal) rhythm.

For several decades, attempts have been made to offer the existence of an endogenous diurnal rhythm and an endogenous annual rhythm as proof of the hereditary nature of acquired characters. However, we must take into consideration the existence of endogenous rhythms having no similarity to external rhythms. Moreover, we should realize the decisive role of the endogenous diurnal rhythms and the endogenous annual rhythms in several extremely important physiological processes. In these cases the endogenous ahythms have a very high selective value. Therefore, the marked predominance of endogenous rhythms showing similarities to external rhythms is readily explainable on the basis of the generally accepted principes of phylogenetic evolution.

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THE PHYSIOLOGY OF CELL DIVISION[1,2,3]

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INTRODUCTION

Any cell has but one of three fates—fusion, division, or death. Fusion is the exceptional event; most cells mature either to divide or to differentiate and ultimately to die. Division is thus the consequence of a special kind of cell history in which the mitotic stimulus is only a proximate cause. Why some cells, like those in the nerves of man, should age until death of their host; why others in the parenchyma of plants or the liver of mammals should function without division unless disturbed; and why those of meristem or embryo should divide in quick succession are questions which constitute the very meat of the problem of division. They are not, however, easily answerable ones. Even if one's attention is restricted to cells undergoing division, in the attempt to characterize conditions leading to it, one finds many events necessary to division but none compelling it. Multiplication of chromosome strands, multiplication of chromosomes, multiplication of nuclei, increases in DNA, increases in energy reserves—events which must precede the replication of cells—occur not uncommonly in the absence of replication. We take this to mean that not the events themselves, but certain relations between them compel the division of a cell; of all the facets of division, however, these relations have been the least explored.

It might be supposed that the chemical and morphological events associated with division could be fitted into a single pattern of intracellular relationships which would have universal application. There are difficulties in achieving this, however, that do not arise from a lack of information on the physiology of division, but follow from certain characteristics of cell organization. The point of importance about these characteristics is that subcellular events, such as those referred to earlier, do not stand in a fixed relation to each other, but vary through the history of the cell.

This absence of fixed relations is part of a general feature of biological

[1] The survey of the literature pertaining to this review was concluded in June, 1955.

[2] The following abbreviations will be used: DNA, deoxyribonucleic acid or deoxypentosenucleic acid; RNA, ribonucleic acid or ribosenucleic acid; ATP, adenosinetriphosphate.

[3] Contribution No. 295 Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa.

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organization in which cell, organism, or field population is characterized by autonomous units, each capable of a limited independence in development and function. Within the cell such units may be identified with the self-reproducing elements familiar to the cytogeneticist, i.e., chromosomes, chloroplasts, oxidative particles, kinetosomes, and sundry other cytoplasmic bodies. How the properties of these elements, and others yet to be discovered are integrated to produce cell organization is a question of the broadest interest in cell physiology. To define what is necessary among the possible relationships between these same elements to produce division is difficult in nature the only demonstrable necessity is that of survival. For cell division this means survival of cell type. In the case of chromosomes, because of their high degree of organization, the requirement obviously imposes strong limitations on modes of behaviour. For the remainder of the cell, the limitations are not at all obvious, and there are no reasonable grounds for assuming that during division only one pattern of relationships can prevail. Indeed, insofar as the development of a cell progressing towards division is concerned, it may be taken for granted that under appropriate conditions any autonomous element can act as a source of physiological control; its activities need only to become limiting to the metabolism of the rest of the cell. How such limiting activities arise is another question, but it is important to note that this possibility makes a single and universal controlling mechanism of division extremely improbable.

There are three levels of relations with which this review is concerned. First, there are the relations embodied by the autonomously duplicating subcellular units which cannot arise *de novo* but must maintain a coordinated multiplication to assure survival of the cell species. Second, there is the basic unit of self-duplication, the cell, without which reproduction of subunits cannot occur, and in which an integration and orientation of activities must take place leading ultimately to its division. Third is the set of relations imposed by the multicellular organism on the individual cell, in which the organism, to assure its survival, controls the occurrence of division in its constituent cells.

THE MULTIPLICATION OF SUBCELLULAR ELEMENTS

Cytoplasmic Components

Subcellular elements—chromosomes, chloroplasts, mitochondria—can multiply independently of cell division. For cytoplasmic components this behaviour is apparent in a number of situations, the most obvious being the response of a unicellular organism, a protozoan for example, to a substantial loss of its cytoplasm (1). Multiplication of particulate elements follows here from a physiological adjustment in nucleo-cytoplasmic relations. An opposite type of effect is achieved by growing *Euglena mesnilii* in darkness. In this case the rate of multiplication of chloroplasts falls below that of the cell and an irreversibly chlorophyll-less organism is ultimately produced (1). A similar situation obtains in yeast with respect to oxidative particles,

where even under normal conditions of growth the progeny of some divisions fail to carry any particles with them, becoming obligate anaerobes by virtue of this loss. Such a loss is often referred to as "cytoplasmic mutation" (3).

Generally, wherever growth occurs, multiplication of cytoplasmic elements must also occur, but the specific conditions favouring the multiplication of a particular cytoplasmic element are difficult to identify. A means of estimating the cellular population of each species of element is required to follow such multiplication, and this is not available, although improvement in cell fractionation techniques may ultimately provide the means. Numbers of chloroplasts or mitochondria are inadequate indices because they do not take into account the proplastids or promitochondria which are morphologically distinct from but genetically identical with their maturely developed forms. Furthermore, unless the cells in question are continuously observed, an increase in numbers resulting from development of proplastids or promitochondria cannot be distinguished from multiplication of mature forms. Doubtless, both may occur and certainly differentiated chloroplasts or mitochondria can undergo division; but the difficulties in following qualitatively and quantitatively the heritable precursors of cytoplasmic elements have restricted observations mainly to unambiguous situations where the particle studied is eliminated from the cell.

Factors leading to such elimination are varied. The genetic constitution of the cell may render its internal environment unfavourable to survival of some of its own organelles; in a mutant of corn, chloroplasts are lost because of their instability in the cytoplasm (4). The effect of darkness in arresting plastid reproduction has already been mentioned. Chemicals such as streptomycin and acriflavin reduce the multiplication rates of chloroplasts in *Euglena* (1) and of oxidative particles in yeasts (3). Obviously, the possibilities of eliminating different cytoplasmic bodies without killing the cells are limited in number. More complete information on the multiplication of cytoplasmic bodies must await experiments with less drastic biological effects. Yet, even so, it is important to note that the available experimental data soundly demonstrate subcellular and cellular multiplication to be dissociable functions.

Chromosomes

More precise information is available about chromosome multiplication, since several criteria can be used to determine number: the counting of strands, the chemical estimation of DNA, and the cytogenetic analysis for degrees of ploidy. The chemical method correlates well with cytogenetic determinations of degrees of ploidy, even though the possibility of small departures from a constancy in the amount of DNA per chromosome set cannot be excluded. The number of microscopically identifiable strands in a chromosome pair is not, on the other hand, strictly comparable with results obtained by the other two techniques. For, with respect to cell division and genetic transmission, the chromosome (or chromatid) behaves as the unit of

multiplication. Within the chromosome, however, the individual strands, each bearing the characteristic linear differentiation of the host chromosome, would appear to be the unit of reproduction. It has indeed been suggested on the basis of recent electron microscope studies that the common unit of reproduction is an "elementary microfibril" of approximately 500 Å diameter (5). It is significant that whichever unit of chromosome reproduction is used as a basis of comparison with the host cell, the multiplication of the one would appear to be dissociable from the division of the other. In the salivary gland cells of *Drosophila* larvae the giant chromosomes consist of multiples of the original thread ranging in number from 500 to 1000 (6), yet these are cells which will undergo no division. In plants, multi-stranded chromosomes ("polyteny") which have arisen, not from an abortion of the mitotic process, but from multiplication unrelated to cell division, are a common phenomenon (7). Some pith cell nuclei in which whole chromosome sets have multiplied without cell division have been calculated to be 128-ploid (8). In many somatic tissues of insects polyploidy is a regular occurrence by virtue of repeated chromosome division without breakdown of the nuclear membrane (9). What regulates such multiplication is unknown; speculatively, it has been associated with the physiological activities of the gene, but the evidence is very slim (7). In some cases, as in the salivary gland cell, there is a striking regularity about chromosome multiplication without mitosis that must obviously arise from a special physiological pattern of the cells concerned. In other cases, as in the root tips of plants, polyploidy would appear to arise from a lack of rigour in physiological control. Much basic information is yet to be derived from electron microscope studies of chromosome structure. If recent observations turn out to have a general application then it could at least be said that for any particular organ the number of "elementary microfibrils" and their arrangement within a chromosome are species specific (5).

Quite a different aspect of chromosome multiplication is presented by studies of chromosome chemistry. Here, interest is in the mechanisms of duplication. Although most information along such lines is restricted to the DNA moiety of the chromosome, there can be no question that it focuses upon problems of basic importance to biology. For, in dealing with the chemical formation of the chromosome in terms of DNA (and to some extent other components), at least three principal questions are introduced: (*a*) To what extent are the structural materials of the chromosome products of chromosome metabolism? Where, in effect, does the autonomy of chromosome duplication find its support in the activities of the cytoplasm? (*b*) How is the structure of the chromosome built? (*c*) What is the chemical basis of self-catalyzed duplication?

(*a*) The specific role of the nuclear apparatus in catalyzing the synthesis of chromosomal components is hardly known. There is no doubt that the nucleus is a center of intensive nucleotide metabolism, but we do not yet understand the significance of the high turnover of nuclear RNA relative to general nucleotide synthesis, let alone to DNA formation (10). There is

evidence to indicate that, within a given nucleus, DNA precursors, at least to the level of polynucleotides, need not originate in the nucleus. The validity of the evidence is, however, not generally accepted. In early studies of marine eggs, it was suggested that there existed in the unfertilized sea urchin egg a nucleic acid of the deoxyribose type which could serve as a source of chromosomal material for mitoses following fertilization (11). Since then, a number of investigators claimed to have demonstrated the existence of cytoplasmic DNA, or a close precursor of it, in eggs of various aquatic forms (12), but the interpretation of their results remains controversial. It has been pointed out that the cytoplasmic DNA of the sea urchin egg could be accounted for by the regular nuclear apparatus plus contamination by small ovarian cells invariably present in egg suspensions (10, 13). This criticism cannot be generally applicable since eggs of *Parascaris equorum*, which can be easily freed from contaminating cells, have 20 times more DNA than can be attributed to the egg nuclei (14). There remains, nevertheless, an appreciable degree of uncertainty about the reality of cytoplasmic DNA stores in unfertilized aquatic eggs. Techniques of DNA determination are probably still inadequate in sensitivity and specificity to measure accurately the very small amount present in the large mass of egg cytoplasm (2).

The idea of an extra-nuclear source of DNA for rapidly dividing cells has also been suggested for plant tissues. Transfer of DNA granules from tapetal cells of the anther to developing microsporocytes has been inferred from cytological evidence (15), but this seems to be without foundation, as tapetal nuclei labelled with P^{32} do not transfer the label to the microsporocytes (16). Indeed, other cytological observations have been claimed to demonstrate that in the microsporocytes of a number of plants Feulgen positive bodies are lost from the nucleus to the cytoplasm (17). The genetic consequences of such losses have not been investigated and the significance of this presumably abnormal behaviour is unclear.

(*b*) The duplication of a chromosome probably involves a simultaneous formation of its protein and nucleic acid components. The conclusion is drawn from autoradiographic techniques applied to root tips, where P^{32} as DNA indicator and S^{35} as protein indicator are found to be incorporated into the chromosomes at approximately the same stage of premitotic development (18). Microphotometric, chemical, and isotope analyses further indicate that the synthesis of DNA is rapid relative to the mitotic interval, and uninterrupted (16, 19, 23). It might be supposed from these observations on simultaneity and relative rapidity that the stimulus to chromosome multiplication abruptly sets in motion a number of biosynthetic mechanisms. This need not be the case, however, since the formation of chromosome substance is not tied to duplication. Although the deoxyribose phosphate chain of DNA appears to be stable, some purine and pyrimidine fractions show variable degrees of turnover when tested with labelled precursors, indicating a breakdown and resynthesis of the side-chains of DNA in the interphase nucleus (20, 21). And at least one protein component of the

chromosome, the "residual protein," shows a degree of turnover comparable with that of cytoplasmic protein (21). This lability of chromosomal substance undoubtedly has significance in the physiological activities of the interphase nucleus, but it also serves to point out that multiplication can result either from a special organization of continuously functioning biosynthetic mechanisms or from a set of mechanisms unique to duplication. Thus far, biochemical studies have no decisive bearing on this point, but they do touch upon it in connection with questions pertaining to the chemical basis of autoduplication.

(*c*) It is generally agreed that the linear order of hereditary units required by genetic evidence must have a material basis in the chromosome. It is controversial, however, whether the properties of the hereditary units can be attributed to a single chemical component of the chromosome. DNA is commonly favoured as the source of linear order for a number of reasons. First, its high degree of structural stability is indicated by the fact that in a nondividing cell the "backbone" deoxyribose phosphate chain undergoes very little breakdown and resynthesis (22). Second, purified and protein free DNA is capable of inducing type-specific heritable transformations in bacteria, a behaviour which is satisfactorily explained as a phenomenon of gene transfer (49). Finally, it is possible from crystallographic data to construct molecular models of DNA compatible with the requirements for self determined duplication (24).

Although the evidence just cited very strongly supports the idea of a unique genetic role for DNA, recent studies of DNA turnover during mitosis are not all in harmony with such a conclusion. The point of issue in these studies is whether or not there is a breakdown of pre-existing DNA in the course of chromosome multiplication. The data, though disturbing to the conception of DNA continuity, are inadequate to compel a different conclusion. If the DNA molecule alone could be labelled to the exclusion of all other components of the cell and its turnover then followed during a determined number of mitoses, an unequivocal answer could be obtained. Such an arrangement, unfortunately, has not been achieved. Measurements of P^{32} incorporated over a given interval into the DNA of rat liver, and accompanying estimations of the number of mitoses occurring during that same interval have been interpreted by means of different methods of calculation. On the one hand, results have been claimed to demonstrate a breakdown of the original DNA molecule to yield two new ones (25, 26); on the other, the data have been interpreted as indicating only a slight breakdown of the deoxyribose phosphate chain (27). In another type of experiment, weanling rats were exposed continuously to $C^{14}O_2$ until their body weight, and presumably their liver weight, had doubled; specific activity of the DNA adenine at the end of the period was found to be half that of the nucleotide pool. On the assumption that the DNA which turns over would have the same specific activity as the pool, it was concluded that the original DNA had not broken down in the course of cell doubling (28). A second assump-

tion which must also be made is that each cell of the original liver undergoes a single mitosis, but there is no evidence for such a pattern of behaviour. The fact that no more than 50 per cent of the DNA-P^{32} of an infective phage culture reappears in its progeny has also been cited as evidence of a breakdown of pre-existing DNA during multiplication (29). But it has been pointed out that the data could be equally well explained by assuming that phage DNA remains intact during replication with a probability that only 50 per cent would become infective again (30). The fallacy of assuming DNA to be the seat of genetic continuity has been argued, again without decision, on the basis of Feulgen staining properties of unfertilized aquatic eggs. That some immature eggs (oöcytes) give a negative Feulgen test has been taken to indicate an absence of chromosomal DNA in what is called the "pronucleus" of the egg (13). In this matter controversy is strong, since the very large size of the nucleus together with a fine dispersion of chromatin material would make a positive reaction difficult to detect. By centrifuging whole eggs, the chromatin has in some cases been concentrated at one end of the nucleus and the DNA then identified by a positive Feulgen reaction (31); in the case of the sea urchin pronucleus a negative result has been reported (13). The demonstration of a disappearance of DNA from the nucleus of the egg would certainly furnish conclusive evidence against DNA being the exclusive seat of genetic continuity. Unfortunately, in this field, data are as equivocal as prejudices are unyielding.

Genetic continuity may be regulated by more than one molecular species. A reciprocity could exist between the components of the chromosome such that any single component could be reversibly removed without interrupting the continuity of the physical pattern. The possibility that "every gene is scrapped at every nuclear division" follows from such a relation (32). The principle of reciprocity is not suggested here as an escape from the experimental difficulties of settling an important question. Reciprocity is an elementary and ubiquitous relation in biological organization; the possibility that it operates in the chromosome (as well as in autonomous cytoplasmic bodies) to the exclusion of a unique genetical species of molecule cannot be discounted.

INTRACELLULAR RELATIONS

In the preceding section the independence of subcellular multiplications from cell division has been stressed. It is, however, clear that with relatively few exceptions the rule of division is perpetuation of cell type, and that such perpetuation requires a net equality in rates of replication for all formed elements of the cell including the cell itself. Causal relations must therefore exist between the duplication of a cell and the corresponding duplication of its autonomous components. The search for such a set of relations is a classic one in cell biology. Students of unicellular organisms have paid particular attention to this question, principally because intercellular factors are not superimposed on the intracellular ones. Much had been made of

the importance of cell size in causing division, the "energid" theory of division recalling names as notable as Sachs and Strassburger. But, apart from focusing attention on the significance of development to cell division, the explanation is of little value since it is inconsistent with fact (5, 33) and is too vague in the light of our present knowledge of cellular processes. It is not that we have at hand a more satisfactory answer to the question, but we do have the possibility of speculating in more specific terms. Thus, a working hypothesis of this sort is here suggested: cytoplasmic components do not multiply in any fixed relation to mitosis; rather their numbers are regulated by certain physiological equilibria in the nondividing cell. Chromosome multiplication is, on the other hand, a precondition to cell division, and the immediate cause of events leading to mitosis is probably to be found in the development of the chromosomes themselves.

Studies of the multiplication of cytoplasmic elements in relation to cell division are few but whatever evidence is available does seem to point in one direction. Use of the phase microscope has made possible a continuous and close observation of mitochondrial behaviour during the division of cultured animal cells (34). From such observation it may be concluded that no special mutiplication of the mitochondria occurs preceding mitosis, and that during mitosis the mitochondria are arrested in function. The arrest is made evident by a loss of normal movement and by changes in morphology that are sometimes extreme to the point of fragmentation or disappearance from microscopic view. Inhibition of mitochondrial movement during division is not a general phenomenon; protoplasmic streaming and mitochondrial movements do not stop, for example, when cambial cells of gymnosperms divide (33). But even in these cells there is no evidence for a multiplication of cytoplasmic bodies preceding mitosis. Descriptions of cell division in a number of algae lead to the same conclusion; even where the chloroplast or chromatophore is a single body, its division generally follows the separation of the chromosomes (35, 36). In *Euglena*, the original plastids segregate and then divide by fission in the daughter cells; in *Spirogyra*, where the morphology of the plastids does not favour segregation, a transverse fission follows nuclear division and the original state of the plastids is restored in the daughter cells by intercalary growth.

Cell division, if it is to result in functional and viable offspring, cannot occur without prior chromosome multiplication. The reason is clear from genetical data. In so far as inheritance is concerned, the chromosome set must be doubled to insure continuity; while on a physiological basis, the whole chromosome set must be transmitted to insure viability. Departures from these requirements are possible only when organ and organism replace the cell as the limiting biological unit, or when polyploidy (or polysomaty) modify the strict requirements of the diploid-haploid relationships. In line with a view long held by cytologists it may be said that the mitotic phase of division is dominated by the chromosome, the remainder of the cell adapting itself to the conditions created by the transitional reorganization of the genetic apparatus.

Mitosis is necessary because of the highly organized structure and limiting number of the chromosome set. No other organelle of the cell shares the same degree of linear differentiation; very few others are represented by so small a number per cell. The requirement for mitosis holds to the extent that these two characteristics of the genetic apparatus prevail. A priori arguments for the universal occurrence of mitosis on the grounds that the living world, like the physical world, must have its universal laws, stem from a naive interpretation of what constitutes the universal in nature. Present controversies about bacterial mitosis would appear to have their origins partly in such a priori notions. Chromosome organization is a product of evolution, not a requirement of life. Whether all bacteria, or blue-green algae, have evolved to a common level of chromosome organization remains to be demonstrated; at the moment, the heat of controversy is sufficient to suggest that the facts are in doubt (37, 38). Subjectively, the writer cannot help remarking that it would be a pity if no living trace were left of a genetic apparatus which once constituted the antecedent to higher cell forms.

Even in plants and animals with a developed chromosome apparatus, the mitotic mechanism cannot be considered as necessary where nuclear division is unaccompanied by division of the cell. For, unless the cell is to divide again, the unequal separation of chromosomes into two or more nuclei, need not have untoward effects on the functioning of the cell. Amitosis may occur in senescent cells, as in *Tradescantia* (39), but in this case the decline in physiological function is cause rather than effect of the absence of a mitotic mechanism. There are a few reported instances were amitosis does occur in well functioning cells. The multinucleate cells of the stoneworts are an example of this type although most coenocytic species develop by mitosis (35). In tissue cultures, chick fibroblasts and osteoblasts and human foetal brain cells have been observed to undergo nonmitotic divisions without impairment of their viability (40, 41). Of even greater interest have been the reports that in human somatic cells the number of chromosomes is not constant, presumably because of a failure in the mitotic mechanism (42). A similar situation has been reported in rodents (43). These results must be accepted with reservation in light of the evidence that inadequate techniques of fixation and staining yield only apparent inequalities of chromosome number (44). Improved procedures, nevertheless, do not rule out the possibility that real variations do exist in the chromosome numbers of somatic tissues. If such variations are real, they furnish an example of the displacement of the cell as the limiting unit in nucleocytoplasmic relations, by the organ or by groups of clustered cells (42).

The Common Sequence of Events During Mitosis

It would be most difficult, if not impossible, to begin a description of the mitotic cycle by designating a universal stimulus to its initiation. To repeat a point, the complexity of cell organization allows for a broad variety of limiting factors, any one of which could be the basis for a particular type of stimulating mechanism. Therefore, without entering into the question of

mitotic stimulation, we might begin our description with a cell in which the chromosomes have already undergone duplication. The separation of chromosome multiplication from the mitotic cycle is, of course, arbitrary, although such a separation has some justification in that the two events can occur separately in nature. Once chromosome multiplication has taken place, three successive sets of physiological relations characterize the mitotic cycle. The first set extends from preprophase to the metaphase aggregation; this part of the cycle has a strong tendency to go to completion once this development is begun. The initial stages of mitosis are difficult to inhibit without some damage to the cell. Animal cells in tissue culture require potent metabolic poisons such as sulfhydryl reagents or sodium fluoride to arrest their development at prophase (45). Plant cells which are inhibited in prophase by nitrogen mustard or protoanemonin, are so affected at the expense of destructive changes in chromosomes or cytoplasm (46, 96). Under natural conditions, interruptions of this phase of the mitotic sequence are extremely rare. They occur in the Opalinid family of parasitic ciliates, where the chromosomes of some species remain in prophase and those of others in metaphase during a large part of the life cycle (47). The questions raised by such behaviour cover not only the identification of factors responsible for thus interrupting mitosis, but also the perplexing problem of how condensed chromosomes function in an interphase nucleus. Generally, the highly dispersed state of the chromosomes in the interphase nucleus is regarded as providing a large surface area for metabolic interactions between chromosome and nucleoplasm. If this view is correct, then for the Opalinids in question, either the manner of chromosome function or the cytoplasmic requirements for nuclear activity must have a pattern unusual in the biological kingdom.

The second physiological period of mitosis begins in metaphase and extends to chromosome separation. It has a less rigid organization. One requirement is general for postmetaphase development, that is, the return of the cell to the interphase condition. The requirement may be fulfilled in a number of ways; commonly it is fulfilled by formation of two daughter cells, but not infrequently by reorganization into a polyploid or a binucleate cell. In the tapetal cells of *Antirrhinum* all three routes are taken (48). Instances of failure to complete mitosis may be found throughout much of the plant and animal kingdoms; tetraploid shoots of tomato or polyploid tissues of insects are familiar examples (9). Sometimes abortion of the mitotic process may prove fatal to the organism. Thus, in *Drosophila* a mutation has been found to be lethal because it inactivates the spindle mechanism in the pupal cells of the larva (50).

The third set of physiological relations overlaps the second and covers the remainder of the division cycle. Its distinctive feature is the return of the cytoplasm as an active influence in cell organization. Cleavage of the cell commonly marks the conclusion of mitosis, but it has only a limited relation to the mitotic cycle; it appears to arise in the cytoplasm as a response to the presence of two nuclear centers. Indeed, the dissociation of

cytoplasmic cleavage from nuclear division is in nature the most common of all mitotic aberrations. It is the basis for the statement that in bacteria, growth and division are independent functions (51). (This is not so in higher organisms where the statement has acquired a different meaning.) This dissociation is also the source of multinucleation, which occurs as a regular form of organization in coenocytic algae or as a variable form in some ciliates. One such ciliate, *Cepedea segmentata*, begins many cleavages but fails to complete them, the result being an elongated, multinucleate cell with an odd segmented appearance (47). In other instances nuclear division and cytoplasmic cleavage become distantly separated in time. Spores of some species of Phycomycetes remain multinucleate up until germination, when cytoplasmic segmentation occurs to produce uninucleate cells (35).

Experimentally, too, it is relatively easy to dissociate cytoplasmic cleavage from nuclear division. Sometimes techniques as simple as the removal of oxygen (52) or the application of hydrostatic pressure suffice (53). Perhaps the most impressive demonstration of the autonomy of the mechanism producing cytoplasmic cleavage is the artificial activation of enucleated amphibian eggs. In one case at least, the development of such an egg to an almost perfect blastula has been described (54). The capacity of the cytoplasm to maintain organized processes in the absence of a nucleus is, of course, limited, and the extent to which it prevails varies widely from species to species, being probably dependent on the products of nuclear metabolism stored in the cytoplasm (2). But, whatever dependence cytoplasmic fission may have on nuclear metabolism, its occurrence would not appear to be directly mediated by the nucleus.

Structural Changes During Mitosis

It is to be expected that the sequence of phases in physiological development would have a counterpart in the structural and metabolic behaviour of the dividing cell. Turning first to the structural changes, three phenomena will be considered: polarity, movement, and cleavage.

Polarity.—Common as polarity is in biological development, we know little of its nature. In mitosis it usually manifests itself in the bipolar spindle apparatus, though not always. In spermatocyte division of some insects a unipolar spindle is formed, all chromosomes aggregating at one pole (55). In root tips of *Drosera* there is no convergence of spindle fibres, and in the spermatocytes of *Nautococcus* the fibres diverge at the poles (45). In pollen tubes of *Lilium regale* mitosis of the generative nucleus proceeds without any spindle formation (56). The source of spindle organization is also none too clear. The spindle is generally believed to arise from some granular elements in nucleus or cytoplasm. In plants the spindle seems, on good evidence, to be entirely intranuclear in origin; in animals this does not always appear to be the case (45, 57). In both animal and plant cells, however, chromosomal fibres appear to form the part of the spindle distal to the poles (57). Whatever the source of the polar elements, spindle formation is

closely associated with the mitotic (or meiotic) development of the chromosomes. It is this association and the evidence cited earlier for the physiological unity of the pre-metaphase stage that give the basis for the introductory statement that the immediate cause leading to mitosis is probably to be found in the development of the chromosomes themselves.

Movement.—The separation of chromosomes occurs by different combinations of autonomous chromosome movement and spindle body contraction (55). In *Lilium* pollen tubes, chromosome movement alone appears to be sufficient to achieve separation (56). In the spermatocyte of *Sciara*, chromosomes aggregate at one pole due to the presence of a unipolar spindle; their subsequent separation has been explained as a self-propelled movement of one complement of chromosomes across the axis of the cell (55). In newt tissues, where the spindle plays its full role in chromosome separation, the process has been followed by phase microscopy. In the first stage of separation there is a steady movement of the chromosome toward the spindle poles, attributable, presumably, to a contraction of the fibrous elements of the spindle. In the second stage, there is an elongation of the interchromosomal parts of the spindle, the distance between chromosomes and poles remaining the same but movement arising from an expansion of nonfibrous elements at the equatorial zone. In the third phase, there is simultaneous moving apart of the chromosomes and cleavage of the cell (58). Thus, a number of mechanisms enter into the overall process of chromosome movement. Recent attempts to explain the process by use of cells obtained from tissue cultures and extracted with glycerol are of limited value. Such "cells" when treated with ATP or other polyanionic reagents contract or extend, simulating to some degree the behaviour of living anaphase cells (59). But this no more than demonstrates the presence of contractile elements within the cell; it does not clarify the nature of the mechanisms necessary to chromosome separation.

Cleavage.—Cleavage of cells is achieved by a variety of mechanisms. In cultured animal cells, anaphase movement of chromosomes and separation of the parent into two daughter cells are closely coordinated (45, 58). In fertilized marine eggs, physical changes of the cortex are primarily responsible for cell separation (45). In plants, the formation of a cell plate midway between the daughter nuclei points to a chemical rather than a physical process, as the cause of cytoplasmic separation (33, 45).

The Metabolic Counterpart of Mitotic Development

The metabolism of a cell may be designated as "mitotic" insofar as it provides substances or energy necessary to division and insofar as its departures from the course of interphase metabolism are coincident with the progress of the mitotic cycle. There are, thus, three headings under which such metabolism may be discussed: (*a*) changes in metabolism induced by mitotic organization, (*b*) provision of substance and (*c*) provision of energy.

XUM

Changes in metabolism.—Commonly with the beginning of prophase there is a marked decline in many metabolic activities of the cell. The change is neither uniform nor general, probably because its occurrence depends upon certain features of nucleo-cytoplasmic relations which vary from species to species. It is obvious that disorganization of the interphase nucleus must have widespread effects on cell behaviour. But, the intensity with which these effects manifest themselves will be governed by a number of factors, none of which we clearly understand. Experiments in enucleation of unicellular organisms offer a good guide to the range of intensities possible. Many ciliates became quickly disorganized upon enucleation, while an alga like *Acetabularia* may maintain its integrity without a nucleus for many weeks (10, 60). Nucleo-cytoplasmic ratio is probably one factor involved since, where the cytoplasm is relatively large, reserves of nuclear metabolic products are more likely to be found.

In staminal hairs of *Tradescantia*, protoplasmic streaming stops during mitosis (45); in the comparatively large and much elongated cambial cells of gymnosperms, streaming continues during division (33). Pollen mother cells of *Trillium* become markedly unstable in sucrose medium once active mitosis begins (61). Mitochondria of animal cells become thin, to the extent of appearing like a string of beads, and sometimes they fragment or even disappear from view (34). In plant microspores, coincident with the development of prophase, there is a steep decline in oxygen consumption, which is probably attributable to changes in oxidative elements similar to the changes observed in animal mitochondria (62, 63). The capacity of dividing amoebae to esterify inorganic phosphate falls during division (64), and this is probably also a result of mitochondrial changes.

In aquatic eggs evidence for a decline in metabolic activities during division has not been found (31). The relationship between chromosomes and cytoplasm in the newly fertilized aquatic egg is, however, singular in the biological kingdom. When cell divisions are synchronous in the egg, and the results of metabolic measurements are applied to individual cells, the ratio of cytoplasm to nucleus is very high compared with most cells. The usual picture of direct nucleo-cytoplasmic interactions could not apply here. This supposition is borne out by the fact that up to the blastula stage, development of fertilized eggs can occur with nonfunctional nuclei or even in the absence of nuclei (31, 54).

Provision of substance.—Compared with other aspects of mitotic metabolism, the biosynthesis of substances necessary to division has been poorly explored. To be sure, chromosomes are duplicated at some time prior to mitosis, and the synthesis at such time of DNA, histones and other chromosomal proteins may be assumed. But, beyond a little information on the biosynthesis of nucleotide constituents, nothing is known about premitotic synthesis of the other fractions of the chromosome. Histochemical evidence is suggestive of changes in chromosome composition during mitosis (5); the

difficulty in deciding whether the changes are apparent or real arises from the fact that staining properties depend as much upon physical state of molecular aggregation as they do upon chemical composition.

Substances which are ubiquitous in the cell—proteins, lipides, ribonucleic acids—have been assayed under conditions of growth and division, but the significance of these measurements is not clear. If the division cycle is regarded as extending from one interphase to the next, then at some points along the cycle all of the cell constituents will be duplicated unless the cell undergoes differentiation. Which of these constituents must be duplicated before division occurs and which following the postmitotic maturation of the cell are fundamental, but yet unanswered questions. Analyses of growth characteristics of chick heart explants indicate a synthesis of protein and phospholipid prior to DNA synthesis, all three substances increasing in concentration before the cells begin to divide (65). One cannot, however, draw the conclusion that this sequence of changes is characteristic for the division cycle of these cells; it is equally, if not more probable that the changes represent a response of the tissue to transplantation. In a number of studies RNA has been considered as a precursor of DNA, apart from its role in protein synthesis (10). In reference to marine eggs especially the idea has seemed attractive since, in the development which follows fertilization, the increase in DNA is matched by a decrease in RNA. The reality of this relationship is, however, doubtful as the decline in measured RNA is due in large part to the loss of jelly coat which behaves like a pentose on acid hydrolysis (10). The demonstration in lily anthers that RNA synthesis follows that of DNA in the preprophase of division makes the idea even more improbable (16). Although recent demonstrations of deoxyribosides in some unfertilized eggs must, for reasons mentioned earlier, be accepted with reservation (10, 13), they do emphasize the possibility that a store of specific precursors for the rapidly forming chromosomes may exist in the egg. Yet even if RNA has no direct role in chromosome formation, its importance in mitotic physiology can hardly be doubted (2, 31). Its intimate association with nuclear metabolism and structure is very well established (31). How this association manifests itself in the course of mitosis is a question which future experiments will surely answer. Some clues are provided by the histochemical demonstration of RNA (and polysaccharide) in the spindle body (31, 52) and of variations in RNA content of the chromosomes during mitosis (5, 66). The suggestion has been made that in the course of anaphase movement ribonucleoprotein is lost from the chromosomes to the spindle body (5, 66). The physiological significance, if any, of such a loss is not at all clear.

Provision of energy.—The first unambiguous demonstrations of the existence of a marked respiratory cycle in association with the mitotic cycle have come from studies of plant tissues (62, 63). Curves of the oxygen consumption of lily or *Trillium* anthers indicate a rise in rate prior to the pro-

phases of microsporocyte meiosis and microspore mitosis, and a fall in rate once division begins. The steep rise in respiration preceding mitosis may be taken to signify an increase in energy requirements of the cell in advance of active division. Such an increase of requirements has, in fact, been shown to occur in epidermal cells of the mouse which fail to divide if deprived of oxygen or oxidizable substrate prior to the onset of division (67). This effect is not attributable to a general deterioration of cell function, as cell viability is otherwise unaffected. If, on the other hand, the cells are similarly treated after entering prophase, their division continues undisturbed. The conclusion here drawn is that energy requirements of mitosis are anticipated before prophase, a conclusion consistent with the respiratory cycle observed in lily and *Trillium* (67). *Chlorella* cells behave in a similar way; they undergo marked phosphorylative activity accompanied by a photochemical liberation of oxygen (the O_2/CO_2 ranging from 2.0 to 4.0) during the "ripening" period preceding mitosis (68).

In fertilized animal eggs, the pattern of mitotic respiration is neither consistent nor clear (31). The existence of respiratory cycles has been much argued, but with no decisive results; fluctuations which do occur in oxygen consumption deviate 10 per cent or less from the mean. In the writer's opinion, generalizations on the nature of mitotic metabolism based on the behaviour of developing eggs have been overdone. At early stages of cleavage, nucleo-cytoplasmic relations within the egg are so singular in nature that a direct and general application of results on oxygen consumption to cells in division can hardly be expected to be meaningful. Other types of experiments have been far more useful in providing information on some general features of mitotic metabolism. Thus, the ingenious use of carbon monoxide to inhibit and light to restore oxidation in the fertilized sea urchin egg has added to the evidence that certain oxidative transformations must precede each prophase in order for division to be completed (69). The fact that respiration in frog eggs may be inhibited 90 per cent without any effect on cell division, is proof enough that even in aerobic organisms reserve substances may yield energy for mitosis without oxidation (31). In general, it may be concluded that some form of premitotic metabolism anticipates the energetic requirements of the cell during active division. In animal eggs, this anticipation may cover several generations; in most cells, it extends only to one. It is difficult, however, to agree with the conclusion that the energetic requirements of mitosis are greater than those of other cellular activities (67); the kind of cell and the kind of activity would be quite important in any such comparison. If oxygen consumption is measured along the axis of the root tip of *Vicia faba*, the region of cell elongation and not that of cell division is characterized by high respiratory activity (70).

It might be supposed, in the light of the suggested role for premitotic oxidations, that in the absence of oxygen, mitosis would go to completion,

once it is initiated. Such a relation does hold for epidermal cells of the mouse (67), but it is not general. In many cell types the requirement for oxygen follows a course similar to that observed for the consumption of oxygen in lily and *Trillium* microspores in which a rise occurs prior to completion of division. In eggs of *Sabellaria*, and less clearly in those of the sea urchin and in animal cells in tissue culture, cytokinesis requires oxygen (31, 52). Some evidence has been presented in favour of the idea that ATP is necessary to completion of cytoplasmic division (71). The use of glycerol models has already been discussed; additional data are derived from experiments in which ATP counters the effect of hydrostatic pressure in inhibiting cell cleavage (72). It is possible that the oxygen requirement of a cell at the terminal stage of division is an inverse measure of the energy stores remaining from premitotic metabolism. Obviously, where mitochondria become active again prior to completion of division, as probably occurs in microspores and animal cells in tissue culture, the energy stores created by premitotic respiration would not need to cover terminal requirements for oxygen.

Oxidation-reduction pathways other than those linked directly to the cytochrome system appear to be necessary to cell division. Glycolysis has been implicated on the grounds of cyclic production of acid in dividing sea urchin eggs (31), and the inhibitory effect of sodium fluoride on the prophase of mitosis supports the idea (45). But, the information available at present does not make clear to what extent cyclic production of acid results from a cyclic arrest of oxidative activity, or to what extent sodium fluoride acts as a block to the production of oxidizable substrate. Studies of filamentous yeast forms have indicated that the capacity of the filaments to prevent the intracellular reduction of tetrazolium dyes is correlated with their ability to undergo division. This property is independent of respiration and appears to be associated with a flavoprotein hydrogen transport system (73). In lily anthers it has been shown that the concentration of ascorbic acid varies cyclically with mitosis (74), and in roots ascorbic acid concentration is greatest in the meristematic regions (75).

The main argument for special oxidation-reduction systems necessary to division comes from the data used by Rapkine to construct his hypothesis of the chemical basis of mitosis (31, 76). Since this hypothesis has provided the principal, if not the sole, concept about which discussions of cell divisions have revolved for the past 25 years, its details may be safely omitted here. Broadly described, soluble sulfhydryl groups are present in relatively high concentration in cells which are undergoing division. In sea urchin eggs the concentration of soluble -SH can be shown to follow a cyclic course synchronous with division. Rapkine attributed this cycle to a reversible denaturation of proteins, and considered the -SH group to be the metabolic regulator of mitotic development. The proposed link between protein denaturation and metabolic activity represented the first attempt

to link structural change with metabolic function in cell division. The role of -SH groups was later broadened to include control of spindle gelation by means of disulfide linkages (31). More recently, the significance of -SH groups to mitotic metabolism has been questioned, and their importance to spindle formation stressed (77), the first because the required high concentration of protein-bound -SH could not be satisfactorily confirmed cytochemically, the second because isolated spindles behaved as though the S-S linkage were important to their integrity. Cytochemical techniques, because of their uncertain nature, are not decisive arguments for or against the importance of -SH groups in division. The cyclic variation in soluble -SH is soundly established from sea urchin eggs by chemical methods. Considering the ubiquity of the sulfhydryl mechanism in metabolic and structural functions of cells, it seems most likely that in division too, the -SH group retains a general significance. That -SH groups play an important role both in enzymatic activities and architectural properties of cells has been amply stressed by a number of writers (31, 76). The flavin catalyzed reduction of disulfide groups in the wall protein of dividing yeast cells illustrates an unexpected role for the -SH group in maintaining the cell wall plasticity necessary for completion of cytoplasmic division (97).

INTERCELLULAR RELATIONS

The Role of Cell Type

In the multicellular organism, intracellular activities associated with division must in some way be controlled. Enough has been said in the preceding sections to indicate that even within a single cell the process of division is in many respects a loosely organized affair. It takes little imagination to see that for the organism, the points of possible mitotic control are many. Mitosis may be inhibited or stimulated in different phases of the division cycle and by different chemical means. Furthermore, the same stimulant or inhibitor may evoke quite different responses, even in contiguous cells, because of the distinctive metabolic poise acquired by each species of cell as the result of differentiation.

It seems superfluous to mention the importance of differentiation in organismic control of division. Yet, particularly in botanical studies, this point is often overlooked in favour of the search for a universal chemical mechanism of mitotic control. It is difficult, because of the heterogeneous organization of plant tissues, to follow the mitotic behaviour of individual cell types. It is certain, however, that a single mitotic stimulus does not produce a uniform response in different parts of the plant. If roots of *Allium cepa* are exposed continuously to 3 p.p.m. indoleacetic acid, the meristematic cells of the tip cease to divide, whereas cells of the pericycle, vascular bundle, and cortex are stimulated to division. Of the cells which divide, those near the root apex respond first; the more highly differentiated and polyploid

cells (up to 8-ploid) respond last. Epidermal cells with small nuclei and pith cells with very large nuclei do not respond at all (8, 78). Physical agents have also been used to demonstrate the importance of differentiation on mitotic behaviour. Thus, if oat seedlings are exposed to light, cell divisions are partially inhibited in the first internode, but are accelerated in the coleoptile and the second internode (79). Even apparently homogeneous tissues may not respond uniformly to conditions promoting cell division. Pieces of pith parenchyma, excised so as to yield a cell population as close to homogeneity as possible, have been followed histologically after being placed in tissue culture medium. In the case of *Helianthus tuberosa*, the whole explant proliferated by cell division and also differentiated. In the case of *Sambucus nigra* only cells at the periphery of the explant divided; of the dividing cells, those in contact with the medium developed homogeneously, whereas those in contact with air tended to differentiate (80). Indeed, the point has been made for some time that the progressive change in character of a cultured plant tissue may be due to unequal rates of multiplication of the different cell types, rather than to a general physiological transformation (81).

In animals, because of the much clearer segregation of cell types into organs, the differential response of cells to mitotic stimulation is obvious. Some cells become so modified in the course of development that they cannot be induced to divide. Thus, the removal of a lobe of liver from a rat stimulates the remaining cells to divide and grow until the liver regains its original state. By contrast, removal of one kidney results in a hypertrophy of the sister organ, to some extent by cell division, but mostly by cell enlargement. Glomerular cells of the kidney are incapable of division (82). Differences in responses have also been noted where the stimulus affecting division arises from a genetic change. In *Drosophila*, a recessive mutation has been described that causes inactivation of the spindle. The inactivation may be observed by cytological techniques at the larval stage of development, but only in the pupal cells, the other cells of the larva having divided normally (50).

Which features of differentiation, it may be asked, are responsible for the different responses of cells to mitotic stimulation that make possible the control of division in the organism? For cells which can no longer divide, an irreversible change in organization has taken place, even if the site of change is uncertain. In the case of cells which do not ordinarily divide but are capable of so doing, the possible mechanisms of control are many. The genetic apparatus, for one, may be limiting by its failure to duplicate. *E. coli* cultures (thymineless strain), if deprived of thymine, are incapable of forming DNA, and do not divide; if thymine is then added to the cultures, the entire population enters into synchronous divisions (83). But failure of DNA duplication (or, for that matter, of chromosome duplication) need not be due to the absence of an essential constituent. In many organisms metabolic relations may block DNA formation, as demonstrated in the lateral buds

of *Tradescantia*, where cells fail to divide because of the inhibition of chromosome duplication by auxin diffusing from the terminal bud (84). Other relations which divert the cell from division may exist. Epidermal cells of the mouse ear are limited in their mitotic activity by energy supplies (67). Still others, like the cultured pith cells of tobacco, are probably limited at a point beyond chromosome duplication where the newly discovered substance "kinetin" acts to stimulate completion of division (85). That the cytoplasm can be the cause of limiting, or stimulating, mitosis has been most interestingly demonstrated in studies of crown gall tumour cells. Apparently, in the normal ancestors of these cells there is an autonomously duplicating cytoplasmic element which, when transformed, renders the cells neoplastic (86).

The Role of the Organic Environment

The organic environment created by an organism for its constituent cells is, in effect, a complex of circulating or diffusing chemicals. The existence of such an internal environment furnishes the basis for what, in plants especially, is termed the chemical basis of growth control (87). To what extent proliferation in a particular tissue is a result of chemical stimulation or to the special organization of the cells involved is difficult, if not impossible, to decide on the basis of what is presently known. In plants the factor of cell type appears to be less important than in animals, but this impression may be due to the greater attention paid to the cellular basis of differentiation in animal embryology. As for the chemicals themselves, how simple or complex their aggregate might be can only be decided in speculative fashion. A simple scheme consisting of auxins and nucleotides has been suggested as the means of growth control in plants (87). That the scheme is plausible is beyond question; that it is a sufficient and necessary one is highly doubtful. Chemical mechanisms, after all, are nature's pawns in the game of evolution. One can point to at least two principal classes of chemical mechanisms by which cell division is controlled in the organism, inhibition and stimulation. But, within these classes, the substances which serve to accomplish the particular effect are many and varied.

Inhibition as a mechanism of mitotic control.—The list of known mitotic poisons is long. Few of them, however, have been investigated from the standpoint of physiological function. In roots of *Avena*, mitotic activity may be correlated with the distribution of several chemically related fluorescent substances. Of these, one, scopoletin, has been reported to be inhibitory to the formation of mitotic figures (88). Its real function in mitotic control is unproven. The fact that scopoletin concentration in the root follows a rising curve beginning at the tip and reaching a peak about nine mm. above the apex is at least not inconsistent with its role as mitotic inhibitor. The weakness of the correlation lies in the fact that cell divisions do not occur more than 2.5 mm. from the tip (89). Auxin produced at the stem apex inhibits divisions in lateral buds; removal of the terminal bud is followed by divi-

sions in the lateral buds unless prevented by application of naphthaleneacetic acid to the tip (84). The action of the auxin in this case is to prevent the duplication of the chromosomes. In starfish ovaries a heat-stable, heparin-like substance is produced which inhibits the maturation divisions of the egg, a necessary inhibition since eggs must be kept from maturing before being shed. It apparently acts by disturbing sol-gel reactions and thus preventing the gelation of the mitotic figure. The compound also acts on eggs of the worm, *Chaetopterus*, but is without effect on mammalian cells (90).

Mitotic stimulation.—The epidermal cells of the mouse ear are affected by a simple environmental factor, glucose concentration of the blood supply. At low glucose levels, premitotic development is arrested. *In situ*, mitotic activity of the epidermis follows a diurnal cycle, the maximum occurring when the mouse is at rest and the minimum when the mouse is subject to muscular activity. Carbohydrate, injected into the bloodstream, will simulate rest. The effect of carbohydrate may also be demonstrated with tissues maintained *in vitro* (67). Attempts to expand this relation into a general mechanism of mitotic control on the grounds that mitosis requires more energy than other cell activities are, in the writer's opinion, tenuous. Too many differences between cell types exist; too many responses to adequate energy supplies are known. In the insect *Rhodinus*, oxygen deficiency appears to stimulate mitosis of the tracheal system at the time of moulting (91), and, in the bean root, cell elongation rather than division is associated with high oxidative (and presumably high energetic) activity (70).

The stimulants to division of plant tissues include a number of different types. There is traumatic acid (1-decene-1, 10 dicarboxylic acid) which acts to renew cell division in the mesocarp of the stringbean pod (92). And, in coconut milk, several factors have been identified which stimulate division in a number of tissues including crown gall tumours and carrot explants (93). Some of the effective components are nitrogenous compounds found in other fruits as well. One of the most active substances belongs to the class of leucoanthocyanins. It occurs as a monoglucoside in the liquid and solid endosperms; the aglucone form which is also present in the endosperm has no activity (94). What particular subcellular process is affected by any of the above compounds in the course of cell development unfortunately has not been clarified. In the case of auxin, when it does stimulate cells to divide, the action appears to be that of promoting chromosome duplication (95). In some plant tissues, for example, callus tissue from the cambial region or the stem pith in tobacco, the completion of the mitotic process seems to be set off by an adenine derivative, "kinetin" (6-furfuryl amino purine). Discovery of this compound is of major interest because of its close association with DNA, and because of its general occurrence in animals and plants. The fact that it is released from DNA *in vitro* by autoclaving suggests a mechanism of partial autolysis by which wounded cells *in situ*, might stimulate neighbouring cells to divide. It remains, of course, to be demonstrated that the structural association between kinetin and DNA is

real rather than an artefact of preparation. Also, the extent to which kinetin is required for the division of different cell types must be determined. In artichoke tissues, at least, the effectiveness of "kinetin" in promoting division is small compared to that of whole coconut milk (98).

CONCLUSION

In all natural phenomena, and so too in cell division, the search is for the necessary at the expense of the contingent. Yet, however we approach the problem, we can find only one necessary condition to serve as point of departure, the necessity of survival. A species of cell survives if the rate of multiplication of itself and of its parts shows a net equality from one generation to the next. Cytoplasmic components do not conform to a rigid pattern during cell divison. Chromosomes, on the other hand, must segregate precisely if cell type is to survive. From this requirement follows the mitotic mechanism. How many of the physiological changes in a cell associated with mitosis are due to a disruption of normal nuclear function, and how many are responses to the needs of chromosome separation cannot easily be decided; the two possibilities are not mutually exclusive. But of the many changes which occur, one can, in summary, arrange them in three categories, each of which must be represented in some form to assure the success of cell division. (*a*) The diversion of biosynthetic systems to the formation of chromosomal and spindle body substance. (*b*) The modification of energetic metabolism to provide a store of energy to serve the requirements of the mitotic phase of division. (*c*) The polarization of cell structure to make possible an equational separation of chromosomes.

How the organism controls the division of its constituent cells is a question which can hardly be answered in summary form. One can only repeat the rather elementary point that cell differentiation as well as the chemical nature of the interior of the organism must be taken into account. Studies of these seem to offer little promise of an ultimate solution of the question in terms of a single and universal chemical mechanism.

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OBLIGATE PARASITISM[1]

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INTRODUCTION

Plant pathologists, plant physiologists, and others have tried unsuccessfully for about 100 years to culture the viruses, downy mildews, powdery mildews, and rusts on nonliving media. Much if not most of this effort has not been recorded in publication. Many regard the task as impossible, and for this reason the term obligate parasites is used to denote these and other organisms which live in such necessary association with living hosts.

Within the fungi alone, all the downy mildews, powery mildews, and rusts are obligate parasites, and these constitute over 5,000 of the some 37,000 species of fungi [Bisby & Ainsworth (29)]. Since all the viruses, most of the chytrids, most parasitic higher plants, and many miscellaneous fungi are also obligate parasites according to our present knowledge, it seems likely that about one fourth of plant parasites are obligate parasites. These obligate parasites include the causes of many of our most important plant diseases.

Importance.—The culture of parasites on nonliving substrata greatly aids our understanding of their nutrition and physiology, but it is not clear at present that culture of obligate parasites of plants will aid in their control. An opposite view has been expressed for obligate parasites of animals [Anon. (7)]. The practical control of plant diseases in the past has depended primarily on exclusion, eradication, chemical protection, immunization, and therapy. Our knowledge of these procedures is about as well advanced for diseases caused by obligate parasites as for diseases caused by nonobligate parasites.

Definition.—When one organism can grow only by securing its food from continued association with another living organism, the condition is termed obligate parasitism [Brown (36), Butler & Jones (40), De Bary & Gurnsey (58), and Gaumann & Brierley (85)]. Obligate parasites therefore are those organisms which can not be cultured on nonliving substrata. The term parasite denotes an organism which derives its nutrition from another living organism, but the distinction between parasites, predators, and symbionts is not a clear one, and the boundaries may be crossed in this review. To culture means to grow indefinitely and to produce new adult individuals similar to those from which development started.

For the culture of one organism apart from all others the term pure culture is commonly used, but pure culture is also used to denote a single species of microorganism grown on tissue cultures of another organism as substrata,

[1] The survey of literature pertaining to this review was concluded in September, 1955.

or for a strain of an organism grown free from all other strains of that organism, as, for example, a pure culture of race 15B of wheat stem rust on living plants in nonsterile soil. Axenic culture has been recently defined [Dougherty (67)] as referring specifically to the growth of one organism free of all others, and this term will be so used in this review. Obligate parasites are therefore those which can not be grown in axenic culture. Several reports of culture involve substrata tissues which are possibly living, such as the culture of the nematode *Neoaplectana* with kidney tissue [Glaser *et al.* (89)], and are therefore doubtful cases of axenic culture.

Obligate parasitism has been otherwise defined. Some have regarded as obligate parasites those organisms which in a state of nature reproduce in association with living hosts. Thus Heald (106) regarded the fungus *Taphrina deformans* as an obligate parasite because of its type of host association, even though he realized it could reproduce by budding in pure culture and in nature. The smuts, ergot, and root nodule bacteria are also sometimes regarded as obligate parasites for the same reason [Gager (80)], but will not be so regarded in this review.

Berkeley in 1846 (24) may have been the first to state the differences in the culturability of parasites. Concerning the fungus *Phytophthora infestans*, he wrote, "I have tried in vain to make the spores vegetate as is so easily done with other species." While *P. infestans* was subsequently cultured on a nonliving substratum, perhaps in 1883 by Brefeld (35) but more certainly in 1900 by Matruchot & Molliard (150), it was recognized as an obligate parasite for many years. De Bary (58) clearly recognized that certain microorganisms grew on living tissues of higher plants but not on dead tissues, and he used the term obligate parasites to denote these organisms. The recognition of obligate parasites as here defined depended on the development of bacteriological culture methods, but obligate parasitism was recognized long before such methods were developed. Most fungi regarded as obligate parasites by De Bary are still so regarded. The Peronosporaceae, the Erysiphaceae, the Uredinales, *Rhizella* and *Polystigma* have still not been cultured, but some of the lichen fungi [Fink (76) and Möller (162)], many of the Chytridiales [Emerson (73)], and at least one species of *Rhytisma* [Schweizer (199)] have since been cultured. In the past, therefore, obligate parasitism has been a fairly stable condition, but several former obligate parasites have since been grown in axenic culture.

The application of bacteriological culture methods to the axenic culture of microorganisms is reviewed briefly by Duggar (71) and Westerdijk (223). Most of the credit for the early work goes to Brefeld (35), the Tulasne brothers (219), and Küster (131). Brefeld, especially, was active in the development of special and natural media for special cases. He clearly recognized that species now regarded as obligate parasites were more difficult to culture than other species, and he recognized that Myxomycetes would not usually grow without the presence of bacteria. According to this reviewer's interpretion, Brefeld indicated in a rather noncommittal way that he had cultured

on nonliving substrata several fungi which subsequent workers have failed to grow in axenic culture.

The list of cultures maintained by the Centraalbureau voor Schimmelcultures at Baarn, Holland (224) is a useful, though incomplete, list of microorganisms in axenic culture.

Scope.—Parasitism and obligate parasitism are represented in associations between many of the main categories of life. The host or the parasite may be either a plant or an animal, or the host or the parasite may not be clearly definable as plant or animal. To take one large category—the fungi—these may be parasitic on bacteria, fungi, algae, nematodes, insects, mosses, ferns, higher plants, and higher animals. Fungi may in turn be parasitized or eaten by bacteria, algae, fungi, nematodes, insects, higher plants, or higher animals. Many, but not most, of these parasitic associations are obligate according to our present state of knowledge. In a review of plant physiology we are naturally concerned mainly with plants and the emphasis in this review will be on microorganisms obligately parasitic on higher plants. The principles involved, however, extend far beyond the scope of this review.

No previous comprehensive review of obligate parasitism has been found, but brief treatments are given by Allen (1), Brown (36), Cutter (56), Dickinson (60), Kern (127), and Zimmerman (254). The principles involved and general aspects will first be presented and then our knowledge of the obligate parasitism of specific taxonomic groups.

The Problem

The spores of most obligately parasitic fungi germinate well in the absence of their hosts, and the germ tubes continue to elongate until the food supply in the spore is exhausted, or until some other growth limiting process sets in. Thereafter, no nutrients are able to promote continued growth. A plausible hypothesis to explain obligate parasitism is that the parasite needs certain labile nutrients which it can get only from the living host. Such labile nutrients might be immediately inactivated by the death of the host [Cutter (56) and Hawker (105)]. Mains (146) has suggested that rusts require certain stereoisomers. Chlorophyll is not necessary for the growth of *Plasmopara* [Morel (164)], *Puccinia* [Sayed *et al.* (196)], or tobacco mosaic virus [White (226)], but rusts usually grow better on green than on chlorotic plants [Cutter (56)] even though the host is apparently well supplied with carbohydrate. The principal problems associated with obligate parasitism are these: Are the parasites concerned really obligate? If so, why? If not, what procedures of culture are most likely to be successful? The position taken in this review is that obligate parasites can eventually be cultured, but that because of inherent differences between them and nonobligate parasites, special techniques will be necessary. A review of past failures and successes should facilitate further advances in knowledge.

The principal limitations in the axenic culture of organisms, and especially of obligate parasites, have been and are: (*a*) the difficulty of separation of the

test organisms from the bacteria and other microorganisms which normally accompany them in nature, (*b*) providing favorable physical environment, and (*c*) providing favorable nutritional environment. Separation of the test organisms from contaminants has been a major problem in the axenic culture of higher animals [Baker & Fergerson (15)], nematodes [Dougherty & Calhoun (68)], protozoa [Cleveland (48)], Myxomycetes [Hok (110)], and Chytridiales [Couch (55)]. Useful methods have involved dilution and washing [Whiffen (225)], differential sedimentation [Cleveland (48)], differential media [Hok (110)], and surface and internal sterilization [Dougherty & Calhoun (68) and Mountain (165)]. Separation of the test organisms from associated contaminants has not been a serious problem in the axenic culture of downy mildews, powdery mildews, and rusts, as greenhouse cultures of these fungi have yielded a high percentage of spores without detectable contaminating microorganisms. If and when a higher degree of freedom from microorganisms is necessary with these fungi, it can be achieved by use of aseptic seedlings [Steinberg (211)] or tissue cultures [Hotson (115) and Morel (164)].

The physical environment of the test substrata has also probably been unimportant as a limiting factor in the axenic culture of most obligate parasites. There is no good reason to believe that the cellular structure of plants plays any specific role in favoring the growth of parasites which inhabit them. Facultative saprophytes, those organisms which normally grow in living plants but which can be grown axenically, usually grow as fast, and in many cases much faster on the artificial, noncellular substrata of conventional culture media than on the living cellular substrata to which they seem well adapted in nature. The maximum rate of increase of *Xanthomonas phaseoli* growing on bean leaves [Allington & Chamberlain (4)] was a doubling of the population in about 5 hours, while in broth [Mason (148)] the population of this organism doubled in about 2.5 hours. Germ tubes of the obligately parasitic *Peronospora destructor* grew at the rate of 50 to 100μ per hour apart from their host [Yarwood (243)] and the obligately parasitic *Puccinia graminis* at about 95μ per hour [Stock (213)]. It is likely that these rates of growth are as high as occur on the living host.

Arens (9) and Schmidt (197) attempted to produce artificial cells with surrounding membranes in the hope of favoring growth of *Plasmopora* and *Phyllosticta*, but gave no clear evidence that this structural modification favored growth. Dickinson (60) believed that agar or fluid surfaces might be injurious to the growth of obligate parasites. He seeded powdery mildews and rusts on artificial membranes but did not indicate that the fungus growth on these membranes was greater than on liquid or agar surfaces. The morphological conditions of plant cells and tissues have never been remotely approached in artificial substrata. If such physical conditions are important for the growth of parasitic fungi, it is surprising that any parasite has been grown in axenic culture.

The axenic culture of organisms, including obligate parasites, can be

resolved into two aspects. The first aspect is the successful culture on any nonliving substrate. The second aspect is the analysis of the nutrient and other conditions necessary for growth. The first may precede the second by many years, as in the use of yeast extract for organisms [White (227)]. With the obligate parasites we are still primarily concerned with the first phase but both phases might be accomplished simultaneously.

The difference between obligate and nonobligate parasites is well emphasized in their response to potato dextrose agar. Most nonobligate parasites make more growth on potato dextrose agar than on water, while most obligate parasites make more growth on water or water agar than on potato dextrose agar. Most nutrient substances which favor the total growth of nonobligate parasites will not even enhance the spore germination of most obligate parasites [Duggar (71)].

Some possible approaches to the problem of a suitable nonliving substrate for obligate parasites are as follows: (*a*) The addition to nonnutrient substrata of specific nutrients which may stimulate the growth of obligate parasites. (*b*) A method of killing host tissues which will neither inactivate their critical growth promoting properties, nor release toxins. (*c*) The production from the obligate parasite of mutant forms which will grow on conventional substrata. (*d*) The extraction from the obligate parasite of the substances necessary for its growth.

All these approaches have been tested, but most emphasis has been placed on the first method. The second would appear most promising.

The obvious procedure of using host extracts as substrata for the axenic culture of obligate parasites has been extensively tested and has been notoriously unsuccessful. Host extracts have been quite useful for tissue cultures of cells of animals but rarely for cells of plants [White (227)]. Even for nonobligate parasites of plants it is rare to secure more growth on host extracts than on potato dextrose agar or other standard media. Most trials have been with extracts from leaf tissues, but phloem and xylem exudates have also been tested. There is even little useful correlation between the susceptibility of the host and the nutritive or toxic value of its sap for pathogens invading the same host. The greater toxicity of extracts from scales of red (resistant) than from white (susceptible) varieties of onions for the pathogen *Colletotrichum circinans* is a unique case in which resistance is localized in dead outer scales [Walker (221)]. The potato fungus, *Phytophthora infestans*, has been grown on substrata containing extracts of several nonhost plants such as oat and lima bean [Clinton (49)], *Cucurbita pepo* [Matruchot & Molliard (151)], and pea [Dickinson & Keay (61)] while extracts of potato have usually been less satisfactory. The lethal action of 0.05 per cent crude phloem exudate from susceptible or resistant hops to the swarm spores of the obligate parasite *Pseudoperonospora humuli* [Newton & Yarwood (170)] is a striking example of the high toxicity of host extracts and the lack of relation of host susceptibility to the nutritive value of host sap. The xylem exudate was practically nontoxic, and crushed leaves were intermediate, but no

extract tested appeared to possess nutritive value. Toxicity of host sap, lack of nutritive value of host sap, or lack of useful correlation between the susceptibility of the host and the response of the parasite to host sap have also been found by Anderson (5), Arens (9), Johnstone (121), Munnecke (166), Parker-Rhodes (175), and others. Ezekiel (74) found host extracts nutritive to *Puccinia graminis* only when very dilute. The toxicity of tissue juices to parasites is not so surprising when it is realized that these same tissue juices may be toxic when applied to the tissues themselves [Prât (183)]. It is obvious that ordinary methods of extracting plant sap are very crude and probably yield many substances which the invading parasite does not encounter in natural infection.

The inability of investigators to recover and demonstrate *in vitro* the nutritive materials known to be present in living plants and necessary for the growth of obligate parasites is one of the most puzzling features of attempts to grow them in axenic cultures. Some of these substances are inactivated by heat. Host stimulation of conidial germination in *Erysiphe polygoni* was destroyed by heating the leaves [Yarwood (239)]. The complete loss of the nutritive value of host tissue for obligate parasites as a result of heat sterilization of the host [Daran (57), Dougherty (66), Johnson & Baker, (120), Matruchot & Molliard (151), and Tenenbaum (216)] would seem to be attributable to chemical rather than to physical changes. The change of nonhost plant tissues from resistant or nonnutritive to nutritive for obligate saprophytes, facultative parasites, and facultative saprophytes as a result of heat sterilization [Coons (52), Green (95), and Rawlins (186)] would also seem to be because of a chemical change. Sterilization of tissues by means of propylene oxide [Hansen & Snyder (101)], nitrogen mustard, or ultraviolet light [Hok (110)] and x-rays [Fassuliotis & Sparrow (75)] should be explored in the culture of obligate parasites. The vitamin inactivating properties of ethylene oxide [Hawk & Mickelson (104)] might be an asset or a liability. Brefeld (35), Dickinson & Keay (61), and Yarwood (241) have noted that extracts from dried plant tissues were nutritive or at least stimulatory, while presumably extracts from similar fresh tissues were not, or were less so.

The importance of phosphorus compounds in obligate parasitism has been emphasized by Allen (1), Cutter (56), Garner & Gottlieb (83), and Atkinson & Shaw (12), and great accumulations of phosphorus in rusted tissues were demonstrated by Yarwood & Jacobson (252). The more luxuriant development of powdery mildews and rusts on hosts well supplied with sugars has been demonstrated by Mains (146), Yarwood (237), and many others, but few direct effects of sugars on these pathogens have been demonstrated.

The empirical testing of nutritive and normally nonnutritive chemicals as additions to substrata for obligate and nonobligate parasites will undoubtedly continue to yield valuable results. Potassium permanganate as used by McKay (153) and Yarwood (243) and the substances known as Tween 60 and Tween 80 used by Dubos (70) and Pelletier (178) are good examples.

It is noteworthy that none or few of the many purified vitamins and other growth substances have been shown to be of value in the culture of obligate parasites of plants [Gottlieb & Hart (92) and Peterson (180)].

Degrees of Obligate Parasitism

Obligate parasitism as defined is an absolute condition, but within the groups of obligate parasites there are undoubtedly degrees of obligateness. The ultimate situation in obligate parasitism would be a parasite which would not germinate without host contact, and after germination would not grow without continuous association with the host. No such case is known to the writer, and, in those cases where germination is stimulated by host contact, stimulation of germination can be induced by means other than a living host. Degrees of obligate parasitism might be distinguishable by the relative amount of growth which different parasites can make without nutrients and by the relative amount of stimulation which can be induced by nutrient treatments other than host contact.

For example, under comparable nonnutrient conditions, the germ tubes of spores of *Erysiphe polygoni*, *Uromyces phaseoli*, and *Peronospora destructor* grow to about 44μ, 390μ, and 500μ, respectively [Yarwood (244)], and on this basis the degree of obligate parasitism would decrease in the order given. Using the best nutrient conditions then known, the lengths of the germ tubes of these three fungi were increased to about 280μ, 1,000μ, and 2,800μ, or the increases due to nutrients were about 536, 156, and 420 per cent. On this basis the ranking in decreasing order of obligate parasitism would be *Uromyces*, *Peronospora*, and *Erysiphe*. If we combine both these criteria and give equal numerical weight to each, the ranking in decreasing order of obligate parasitism would be *Erysiphe*, *Uromyces*, and *Peronospora*. The writer believes this latter ranking may represent the true order of things—that is, *Peronospora* will be most easily cultured, then *Uromyces*, and then *Erysiphe*. This method of comparison of degrees of obligate parasitism is of doubtful value without more data. Viruses, since they have as yet exhibited no confirmed tendency to increase apart from living tissue, will probably be more difficult to grow in axenic culture than any of the fungi.

Comparison with Parasitism in Animals

Obligate parasitism is probably basically similar in plants and animals, but there are some important differences. Viruses are obligately parasitic in plants and animals but no case is known in which the same virus is parasitic in both plants and higher animals. A few cases are known of viruses which multiply and cause disease in plants and multiply but do not cause disease in insects [Black (30)]. One case is reported of a virus which multiplies in man and is recoverable in a viable condition in plants [Murphy & Syverton (167)]. Several plant and animal viruses have been maintained in pure tissue (monoxenic) culture [White (227)] but none in axenic culture. There are cases of viruses which exist as obligate parasites of plants or of animals without causing recognized disease [Smith (204)], and facultative parasites may occur

in healthy plant tissues [Lutman & Wheeler (140) and Robbins & Hervey (192)].

Rickettsias are obligately parasitic in animals [Breed *et al.* (34)] but none is known in plants. Most of the obligate parasites of man other than viruses are protozoa [Hall (97)]. There are few protozoa parasitic on plants and these may be presumed to be obligate parasites. In a few cases the same flagellates are believed to be parasitic in insects and in plants [Holmes (111)]. The obligate parasites of plants, other than viruses, are mostly fungi. Fungi are not uncommon as parasites of higher animals, but few of these have resisted axenic culture [Wolf & Wolf (232)]. The fundamental differences in the morphology and physiology of plants and animals have not been clearly related to the behavior of obligate parasites in these contrasting types of hosts.

Evolution, Adaptation, and Mutation

Obligate parasitism is commonly regarded as an evolved state of host adaptation which has progressed through the stages of obligate saprophytism, facultative parasitism, facultative saprophytism, and perhaps symbiosis [Butler (38), Kern (127), Melhus & Kent (157), and De Bary & Gurnsey (58)].

While symbiosis is a type of parasitism, its place in this evolutionary scheme is not clear. Symbionts are more advanced than obligate parasites in that they cause less damage to their hosts or may stimulate the total growth of their hosts, but less advanced in that many of them can be grown in axenic culture. The same fungus (*Armillaria mellea*) may be a facultative parasite with respect to certain hosts [Heald (106)] and a symbiont with respect to others [Hamada (98)], or the same bacterium (*Rhizobium leguminosarum*) may be symbiotic under certain conditions and as certain strains and injurious under other conditions or in other strains [Thornton (217)].

Obligate saprophytes are those organisms, such as *Pyronema confluens* and *Neurospora sitophila*, which must secure their nutrition from dead organic matter. Facultative parasites are organisms, such as *Rhizopus nigricans* and *Botrytis cinerea*, which normally live as saprophytes but may attack living tissue, especially senescent tissue or storage organs. Facultative saprophytes are organisms, such as *Venturia inaequalis* and *Phytophthora infestans*, which normally occur as parasites but which can grow saprophytically.

Through association with senescent vegetation some obligate saprophytes may have acquired the property of attacking living tissue, and have become so adapted to it in the sequence indicated as to become obligate parasites. This type of evolution has not been satisfactorily established. If ontogeny recapitulates phylogeny, the common germination of spores of obligate parasites in the absence of their hosts might support the idea that obligate parasites have evolved from nonobligate forms. The early work of Massee (149) in developing a parasitic *Trichothecium* from a saprophytic form has not been adequately confirmed in detail or in principle, but limited degrees of adaptation seem well established.

Adaptation in plant pathogens has been reviewed by Christensen & Daly (47) and Stakman & Christensen (210). It is commonly, but not necessarily true that pathogenicity of nonobligate parasites is weakened by maintaining them in axenic culture, and restored by host passage [Bernard (25), Bonar (31), Frick (79), Kelman & Jensen (126), Lilly & Barnett (136), McKeen (154), and Milstrey *et al.* (160)]. With obligate parasites, pathogenicity for one host may be weakened or increased by passage through another host. Some of these cases are apparently due to separation of mixtures of strains of the pathogen, but others appear to be changes induced in a single strain as a result of host passage [Anon. (8), Bawden (19), Berks (23), Bertani & Weigle (26), De Bruyn (59), Gale & Davies (81), Holmes (114), and Smith (203)]. The nature of adaptations in pathogens and other microorganisms is a subject of much controversy [Christensen & Daly (47) and Gale & Davies (81)], but it is noteworthy that adaptation is most credited in bacteria and viruses. While the nature of these inherited changes in pathogenicity is fundamental to the study of obligate parasitism, the extensive literature on the subject is considered far too great to be covered by this review. This reviewer regards slight but progressive changes in the hereditary properties of organisms induced by the substrata as a real possibility in bringing about changes from obligate parasitism to facultative saprophytism and vice versa.

If these changes were occurring in nature one might expect to find transitional stages, but such stages are exceptional. With viruses, powdery mildews, and rusts, no close relatives among the facultative saprophytes are known. In the rusts, Jackson (119) and Savile (195) have indicated that obligate parasitism may be a primitive condition, and the closest facultatively parasitic relatives of the rusts may be the smuts. In the Peronosporales, *Phytophthora infestans* and *P. phaseoli* appear intermediate between the facultatively parasitic members of the genera *Pythium* and *Phytophthora* and the obligately parasitic Peronosporaceae. For this reason comparative studies of *P. infestans* with members of the Peronosporaceae might yield valuable information on the evolution and nature of obligate parasitism.

Adaptation of obligate parasites to nonliving substrata seems a plausible approach to their axenic culture. Hotson & Cutter (116), with the rust *Gymnosporangium*, and Hok (110), with the slime mold *Physarum*, explain their reported success in the axenic culture of these forms as probably due to mutation or adaptation. Yarwood (243) failed to detect any adaptation of *Peronospora* to agar in four generations.

Mutation may play several roles in obligate parasitism. The adaptation of obligately parasitic fungi to nonliving substrata [Hotson & Cutter (116); Hok (110)] and the adaptation of saprophytic fungi to parasitism on hosts not previously parasitized [Massee (149)] could, if true, each be due to mutation of the pathogen. Mutations generally represent loss of characters [Beadle & Tatum (20)], including loss of pathogenicity [Keitt & Langford (125), Christensen *et al.* (46), and Garber & Hackett (82)], but may involve development of characters not previously present. Loss of ability to synthe-

size choline has been correlated with loss of pathogenicity in *Venturia inaequalis* [Boone *et al.* (33)]. The production of obligate parasites by induction of mutations for deficiencies in unstable nutrients found only in the living host is a possibility. Stakman & Christensen (210) indicate mutation rates as high as 0.8 per cent for parasitic fungi; some of these mutations can be expected to affect the culturability of the species.

Characters Associated with Obligate Parasitism

Obligate parasitism is usefully correlated with several other characters of organisms, and has been reported to be associated with several more. While it is usually not clear whether these correlations are causal or accidental, some of them will be briefly reviewed.

Intracellular existence.—Most obligate parasites are intracellular, at least in part. With viruses [Bawden (19)] and chytrids [Fitzpatrick (77)], the parasitic stage is usually entirely intracellular. All members of the Peronosporaceae and Uredinales have intercellular hyphae and intracellular haustoria. The Erysiphaceae have mostly superficial hyphae and intracellular haustoria.

However, many nonobligate parasites are also intracellular. The easily cultured root nodule bacteria are normally intracellular [Fred *et al.* (78)]. Haustoria are found in the facultatively saprophytic *Pythium palmivorum* [Butler (37)], *Phytophthora infestans* [Butler & Jones (40)], *Coccomyces hiemalis* [Higgins (109)], and in many other fungus species. The presence of haustoria in *Phytophthora infestans* and their absence in most easily cultured species of *Phytophthora* might suggest a correlation between the presence of haustoria and the degree of difficulty of culture within a genus, but *P. paeoniae* has haustoria and is easily cultured [Cooper & Porter (53)]. The occurrence and significance of haustoria in many groups of fungi are reviewed by Rice (189). Many of the fungi listed by Rice are presumed to be facultative saprophytes, but, with many, axenic culture may not have been tested. Enough have bene tested however, to make it clear that the presence of haustoria is not a reliable indication of an obligate parasite.

Haustoria are commonly considered feeding organs. Butler & Jones (40) and Brown (36) have suggested that haustoria may be specifically adapted for the uptake of proteins from the interior of plant cells and that these proteins, which are necessary for the nutrition of the parasite, can not be otherwise absorbed by its mycelium. This is pure speculation. By comparison with dodders and mistletoes, the concept of haustoria as feeding organs seems reasonable, and the writer believes that the haustoria of powdery mildews undoubtedly function for food absorption, though even here there is evidence (to be presented later) that the parasite can derive nourishment from the host without haustoria. With the intercellular downy mildews and rusts, however, there is little objective basis for regarding the haustoria as feeding organs.

It would be hard to argue that the intercellular nonobligate *Taphrina*

deformans and *Epichloe typhina* without haustoria [Butler & Jones (40)] are any less successful parasites than the intercellular obligately parasitic *Peronospora destructor* or *Puccinia graminis* with haustoria. Bernard (25) has suggested that haustoria are formed as a consequence of the resistance of the host to the pathogen. Further evidence that haustoria are not necessary as feeding organs is the negative observation that *Phytophthora infestans*, which forms haustoria in potato tubers [Butler & Jones (40)], does not form haustoria in tomato fruits, and that *Phytophthora infestans*, *Coccomyces hiemalis*, and *Ustilago avenae*, which usually form haustoria in their hosts, do not form haustoria in axenic culture.

Appressoria and substomatal vesicles.—The germ tubes of the downy mildews, powdery mildews, and rusts commonly form appressoria prior to host penetration. Downy mildews and rusts also commonly form substomatal vesicles in the substomatal chambers of their hosts prior to the formation of infection hyphae. It might be thought that these structures were necessary antecedents to further vegetative growth by these fungi, and that if their formation could be induced in artificial culture, further axenic growth would be more likely. Hurd-Karrer & Rodenheiser (117) and Sharp & Smith (201) report they were able to stimulate the formation of appressoria and substomatal vesicles of *Puccinia graminis* with added nutrients and zinc, but they do not report that the formation of appressoria and substomatal vesicles favored further vegetative growth. Many nonobligate parasitic fungi in nature form appressoria prior to penetration, but do not necessarily form them in successful axenic culture. There is, therefore, no good reason to believe that the formation of appressoria or substomatal vesicles is necessary or desirable for successful axenic culture of organisms which form these structures in nature.

Taxonomic groups.—There is little apparent relation between obligate parasitism and the taxonomic position of the parasite. The viruses, with an uncertain taxonomic position, the Chytridiales, Albuginaceae, and Peronosporaceae in the Phycomycetes, the Erysiphaceae in the Ascomycetes, the Uredinales in the Basidiomycetes, and the miscellaneous obligate parasites, are no more closely related to each other than they are to many nonobligate parasites. Obligate parasitism obviously arose independently on several different occasions [Kern (127)]. Within the above groups it is quite possible that the many examples evolved from more primitive obligate parasites within the group, but there is no reason to believe that obligate parasitism as such played any part in this later evolution.

Most species in the taxonomic groups considered to comprise obligate parasites have undoubtedly not been tested in axenic culture, but these species are regarded as obligate parasites by analogy. It is probable that different species within each group will show differing responsiveness to nutrient treatments. For example, *Erysiphe graminis* has shown greater responsiveness to nutrients than has *Sphaerotheca lanestris* [Yarwood (241)]. When a really successful nutrient treatment is discovered, however, it is

likely that it will apply to many species within the group. For example, alkaline egg yolk has been found to increase the germ tube length of all powdery mildew species with which it has been tested.

Host reaction.—Obligate parasites usually have a benign, nonnecrotic or even stimulatory effect on their hosts. Hypertrophy may be induced by viruses, chytrids, and members of the Albuginaceae and Uredinales. This is in contrast to the necrotic reaction usually caused by facultative parasites and facultative saprophytes. The Peronosporaceae present an evolutionary sequence. With the facultative parasites, such as *Phytophthora nicotianae* and *P. citrophthora*, there is usually an outright killing of the host tissues. With the facultatively saprophytic *Phytophthora infestans*, there is a slightly delayed necrosis, and sporulation usually occurs in living tissue. In the obligately parasitic *Plasmopara viticola* and *Peronospora destructor*, there may be no apparent necrosis until several days after the sporulation of the pathogen. These differences between obligate and nonobligate parasites which are reflected in gross appearances are similarly reflected in cytologic studies [Rice (190)].

However, many exceptions to these generalizations are known. The host-parasite association and hypertrophy caused by the facultatively saprophytic *Agrobacterium tumefaciens* and *Taphrina deformans* are as benign as with most obligate parasites. The effects of the obligately parasitic tobacco mosaic virus on bean and of the tobacco necrosis virus on cowpea are as necrotic as those of any facultative saprophytes in their hosts. These two cases may be correctly considered to be resistant reactions of the hosts, but, if so, they are of an entirely different order of resistance from the necrotic reactions (hypersensitivity) of certain hosts resistant to powdery mildews and rusts. With the virus-host association mentioned and with others which might be mentioned, there is an extensive multiplication of the virus pathogen, apparently almost simultaneously with the manifestation of the necrotic reaction [Yarwood (248)]. On the other hand, with the severe necrotic reactions due to fungus obligate parasites, the pathogen usually does not grow to a stage from which it can be re-isolated.

Stimulation by host before penetration.—Host stimulation of germination or growth has been reported for the obligately parasitic *Erysiphe polygoni* [Yarwood (238)], *Heterodera rostochiensis* [Mai & Spears (145)], *Melampsora lini* [Hart (103)], *Cronartium ribicola* [Colley (51)], *Orobanche ramosa* [Isard & Hitier (118a)], mycorrhizal fungi [Melin (158)], and the nonobligate *Urocystis tritici* [Noble (172)] and *Botrytis cinerea* [Brown (36)], and is therefore not clearly a characteristic of obligate parasitism. De Bary & Gurnsey (58) and Duggar (71) have indicated that nutrients in the substrata are less necessary for the germination of spores of obligate than of nonobligate parasites. The ultimate in obligate parasitism would seem to be the necessity of contact of the parasite with the host at all stages of development of the parasite including spore germination. However, those obligate parasites which are favored by host contact during spore germination do not exhibit

any higher type of parasitism than those forms with spores which germinate well without host contact.

Host vigor.—Obligate parasites as a group are usually favored by young, well nourished, well watered, rapidly growing, vigorous host plants [Butler & Jones (40) and Corner (54)], while nonobligate parasites are less likely to require a vigorous host [Bennett (22) and Yarwood (237)]. There are too many exceptions, however, to make this a useful generalization. The luxuriant growth of bean powdery mildew on plants stunted by low soil moisture [Yarwood (245)] and the greater multiplication of tobacco mosaic virus on bean leaves at low than at high carbohydrate level [Yarwood (248)], are two such exceptions.

Thigmotropic response.—Dickinson (60) has shown that most of the germ tubes of certain downy mildews, powdery mildews, and rusts grew away from the water, agar, or gelatin surfaces on which they were resting, while germ tubes of most other fungi grew along the surface on which they were lying or into the substrate. However, Dickinson does not state that the germ tubes which grew along the surface of the substrate made any greater or less growth than those which grew away from the substrate. With the powdery mildew, *Erysiphe polygoni*, the direction of germ tube growth may be completely determined by light [Yarwood (236)] and germ tubes which grow into the air may actually grow much longer than those which strike the substrate and form appressoria. The fact that germ tubes of powdery mildews show a marked response to nutrients [Yarwood (241, 244)] without regard to thigmotropic response might indicate that thigmotropic response was not a critical factor in the axenic culture of powdery mildews.

Physiologic specialization.—It is commonly believed or inferred, and with good reason, that physiologic specialization is more characteristic of obligate than of nonobligate parasites [Brown (36), Butler & Jones (40), and Stevens & Stevens (212)]. This belief arises partly from the intensive study and large number of physiologic races of the obligately parasitic *Puccinia graminis* [Stakman & Christensen (210)] and partly because physiologic specialization is the only kind of strain variation commonly studied with obligate parasites. The greatest number of cultural types of a single species is about 10,000 for the facultative saprophyte *Ustilago zea*. Physiologic specialization based on host reaction has also been extensively demonstrated in the nonobligate *Phytophthora infestans* [Niederhauser et al. (171)], *Colletotrichum lindemuthianum* [Schreiber (198)], and *Corticium solani* [Le Clerg (133)].

Host range.—Host ranges for obligate parasites are usually smaller than for nonobligate parasites [Brown (36), Gaumann & Wynd (86), and Hawker (105)], but many exceptions to this rule are known. The obligately parasitic *Erysiphe polyphaga* [Hammarlund (100)] has a much wider host range than the facultatively saprophytic *Colletotrichum lindemuthianum* [Barrus (17)]. Usually the hosts of a given obligate or nonobligate parasite are closely related, as, for example, the uredinial stage of *Puccinia graminis* on grasses [Arthur & Cummins (11)], but, with the obligately parasitic viruses such as

tobacco mosaic virus and aster yellows virus [Holmes (113)], the multitude of hosts may be widely separated taxonomically.

Heteroecism.—The well-known heteroecism of many rusts [Arthur & Cummins (11)] and a few viruses [Black (30)] might suggest that heteroecism is a characteristic of obligate parasites. The less well known and less well defined heteroecism of *Sclerotinia heteroica* [Woronin & Newaschin (234)] and *Cicinnobolus cesatii* [Yarwood (240)], if confirmed, would weaken this idea.

Hypersensitivity.—The term hypersensitivity was first used in plant pathology by Stakman (209) to indicate the rapid death of cells of resistant hosts when attacked by *Puccinia graminis.* The term has since been applied to other obligate parasites such as other rusts, powdery mildews, and viruses. Some writers [Butler & Jones (40), Chester (44), and Wingard (230)] refer to hypersensitivity as a type of extreme susceptibility in which the host cells are so susceptible to infection that they are killed, and the obligate parasite, which depends on living cells for its nutrition, dies of starvation. This reviewer does not regard hypersensitivity as a type of extreme susceptibility, nor does he consider it uniquely characteristic of infection by obligate parasites. What is essentially the same reaction, but associated with resistance to nonobligate parasites, is produced by infection with *Botrytis* [Wilson (229)], *Cladosporium* [Bond (32)], *Colletotrichum* [Leach (132)], and *Sclerotinia* [Loveless (138)], and perhaps many other fungi.

Acquired immunity.—Acquired immunity to plant pathogens is most easily demonstrated and best recognized with obligately parasitic viruses [Price (184), Gilpatrick & Weintraub (88), and Yarwood (249)]. It is claimed but not confirmed with three rusts [Yarwood (250)]. With nonobligate parasites it is claimed with infections of *Rhizoctonia* [Bernard (25)] and *Rhizobium* [Virtanen & Linkola (220)]. If these latter claims are confirmed, it may be said that plants may develop acquired immunity to nonobligate parasites as well as to obligate parasites.

Degeneration and parasitism.—Various types of degeneration are associated with parasitism of animals [Lull (139)]. It might therefore be expected that with obligate parasites of plants this reduction and simplification of structure would be greater than with nonobligate parasites. No clear evidence of this has been found. If viruses are organisms, they are probably about as reduced and degenerate as can be imagined, but, since the existence of saprophytic viruses is not clearly established, there is little basis here for associating obligate parasitism with degeneration. With bacteria, no morphological and few physiologic differences are known between saprophytic and parasitic forms and no obligately parasitic forms are known. With fungi perhaps the only basic difference between saprophytic forms and forms parasitic on plants is that some parasitic forms have haustoria, but even this character does not distinguish obligate from nonobligate forms.

In plant pathology it is only with parasitic higher plants that there is clear degeneration associated with parasitism [Kern (127) and MacDougal & Cannon (143)]. The parasitic forms are believed to have evolved from the

free living forms, and, associated with their adaptation to parasitism, there has been a reduction in roots, leaves, and chlorophyll.

Metabolites.—Gaumann (84) has stated, and it seems reasonable to believe, that most symptoms of plant diseases are caused by metabolites of the pathogens. A striking evidence of this is the killing, injury, or stimulation of the host in advance of the pathogen. According to Bail (13), Bessey (28), Brown (36) and MacDougal (142), there is less evidence of metabolite production by obligate than by nonobligate parasites. That metabolites are produced by obligate parasites, however, may be indicated by the odor of pycnia of rusts [Plowright (182)], by the translocated effects of powdery mildews [Allen (1) and Yarwood (246)], by the self-stimulation [Ezekiel (74)] and self-inhibition [Allen (2) and Yarwood (250)] of rust uredospores, by the formation of ammonia by rusts [Gretschushnikoff (96)], and by the formation of nonvirus protein in infections by tobacco mosaic virus [Takahashi & Ishii (215)]. Viruses are unique in that respiration of purified viruses is usually not detectable [Andrewes & King (6) and Maramorosch (147)], but respiration has been demonstrated for the obligately parasitic powdery mildews [Allen & Goddard (3)] and rusts [Shu *et al.* (202)].

Taxonomic Groups of Obligate Parasites

Bacteria.—Obligate parasites are recognized in all the main categories of plant pathogens except the bacteria. While association of bacteria with higher plants in nodules represents a high degree of symbiosis, all the causal bacterial forms are believed to be easily cultured on simple, nonliving, artificial media. Among the bacteria there is no apparent relation between type of parasitism and ease of culture on nonliving substrata. Symbiotic and hyperplasia-producing forms such as the root nodule bacteria and crown gall bacteria are about as easily cultured as forms causing outright necrosis such as the bacteria causing fireblight or angular leaf spot [Elliott (72)]. Obligately parasitic bacteria are believed to exist intracellularly in insects [Meyer (159)].

Viruses.—To those who regard viruses as chemicals it may seem superfluous to consider the obligate parasitism of these infective entities. But many regard viruses as organisms and, to these workers, viruses are obligate parasites. Mayer (152), who was the first to demonstrate the infective nature of tobacco mosaic virus, was probably the first to provide evidence of its obligate parasitism. He wrote, "I tried to isolate these questionable organisms by Koch's method and other methods; . . . none of these, used as inoculum, were infectious to healthy tobacco." Iwanowski (118), Beijerinck (21), Goldsworthy (90), and Grainger (94) have confirmed that the cause of tobacco mosaic could not be cultured on the usual or on special, nonliving media.

Reports of the axenic culture of viruses [Krueger & Baldwin (128), Kunkel (130), and Olitsky (173)] have not been adequately confirmed. The finding by Holmes (112), Samuel & Bald (193), and Yarwood (247) of an increase in

infectivity of tobacco mosaic virus *in vitro* with time, could be interpreted as due to the increased infectivity of the original number of virus particles by disaggregation or by some other mechanism. The association of bean mosaic virus with otherwise axenic cultures of *Xanthomonas phaseoli* [Hedges (107)] has apparently not been confirmed.

Myxomycetes.—Myxomycetes (slime molds) are usually not considered parasites, but their association with the microorganisms they ingest as food is similar to that of obligately parasitic fungi on higher plants. Bacteria are commonly considered necessary for the normal development of members of this group. [Raper (185) and McLennan (155)]. For many years no one succeeded in the axenic culture of a slime mold, though Brefeld (35) indicates he may have cultured *Polysphondylium* without bacteria. Cohen (50) failed to grow several species on oat agar in the absence of other microorganisms but grew some on an agar substrate with living or autoclaved yeast. Hok (110) was unable to maintain normal growth of 17 isolates of slime molds on autoclaved yeast but secured vigorous growth on yeast which had been killed by nitrogen mustard or ultraviolet radiation. Here, as with certain obligately parasitic fungi of higher plants, heat partially destroyed or rendered unavailable the growth-promoting metabolites of the host. Hok was able to isolate a variant strain of *Physarum polycephalum* which was able to grow well on autoclaved yeast.

Chytridiales.—Most parasitic chytrids are believed to be obligate parasites, but perhaps because of their relatively low order of economic importance, few cultural studies of them have been reported. The economically important *Plasmodiophora brassicae* has been reported grown in axenic culture by Jones (122) and *Spongospora subterranea* by Kunkel (129), but present authorities [Karling (123)] appear to doubt this. According to Emerson (73), only two of the 300 or more described species of Chytridiales had been cultured axenically prior to 1940, but in the ten succeeding years many were grown in bacteria free cultures. With the species which have been cultured axenically, the main difficulty appears to have been the mechanical separation of the chytrid from the accompanying bacteria, rather than the fastidious appetite of the chytrid.

Peronosporales.—The Peronosporales constitute the best, long-standing example of obligate and nonobligate parasitic fungi in the same or closely related genera. All members of the Albuginaceae and the Peronosporaceae have haustoria and have not been cultured. In the Pythiaceae, many members of the genus *Pythium* have been cultured (224) but the report by Harrison (102) that *Rhizophagus* is a *Pythium* is still ambiguous. Within the genus *Phytophthora* are species without haustoria which are readily cultured on standard media, species with haustoria which are cultured with difficulty only on special media, and at least one species, *Phytophthora Cyperi-rotundati* [Gibson *et al.* (87)], which has thus far resisted culture.

The species *Phytophthora citrophthora*, *P. infestans*, and *Peronospora destructor* may be used as representatives to trace the evolution of parasitism

from facultative parasitism through facultative saprophytism to obligate parasitism (Table I). This evolution of parasitism is nicely correlated with parallel changes in the mycelia, the haustoria, the sporangiophores, germination of sporangia, dissemination of sporangia, and host reaction. Because transition from facultative parasitism to obligate parasitism appears to occur in such obviously closely related forms in the Peronosporales, it would seem that studies of obligate parasitism might be more profitably studied here than in the viruses, the Erysiphales or the Uredinales, where such transition forms are not known to occur. Other species of *Phytophthora* which have been difficult to culture have been *P. phaseoli* [Leonian (134)], and *P. fragariae* [Hickman (108)].

Phytophthora infestans is an especially informative species because it was for many years regarded as an obligate parasite. It was perhaps first cultured by Matruchot & Molliard (150, 151), who used boullion of *Cucurbita pepo* as a substrate. There have been reports by Clinton (49), Dickinson &

TABLE I

CHARACTERS CORRELATED WITH PARASITISM IN THE PERONOSPORALES*

Character	Species		
	Phytophthora citrophthora	*Phytophthora infestans*	*Peronospora destructor*
Mycelium	Inter- and intracellular	Intercellular	Intercellular
Haustoria	None	Rudimentary	Specialized
Sporangiophores	Unspecialized	Specialized, indeterminate, evanescent	Specialized, determinate, long lasting
Position of sporangiophores	Aquatic	Aerial	Aerial
Germination of sporangia	Principally indirect	Principally indirect	Direct
Host reaction	Necrosis	Delayed necrosis	No necrosis
Parasitism	Facultative parasite	Facultative saprophyte	Obligate parasite

* Information principally from Fitzpatrick (77), Butler & Jones (40), Smith (205), Tucker (218), and Yarwood (243).

Keay (61), D'Oliveira & de Mouros (65), Payette & Perrault (176), Pethybridge & Murphy (179) and Snieszko *et al.* (206) of improved or special media for this species.

The greatest recent recorded success in the culture of a true downy mildew is that of McKay (153) with *Peronospora destructor*. McKay stimulated germination of oospores with potassium permanganate, and secured as

much as 15 mm. of hyphae from such oospores in six days on potato dextrose agar plus onion sap. The type of growth was not what would be expected of a downy mildew. Septa were formed in abundance and structures like *Oidium* conidiophores bearing dark chlamydosopre-like bodies were formed. By contrast, the greatest growth of *P. destructor* secured by Yarwood (243) was 7 mm. of hyphae, and no septa, conidiophores, or chlamydosopres were formed. Yarwood found that potassium permanganate, dipotassium phosphate, glycerine, cysteine hydrochloride, potato dextrose broth, asparagin, riboflavin, potassium nitrate, sucrose, calcium glycerophosphate, histidine, glutathione, tocopherol phosphate, and extracts from young cultures of *Rhizopus nigricans* and *Phytophthora citrophthora* increased germ tube growth. Daran (57) indicates that germ tubes of *Sclerospora maydis* from sporangia reached several millimeters in length under the influence of host extracts and certain nutrient chemicals.

Erysiphaceae.—Powdery mildews differ from most other fungi in their superficial hyphae, their tolerance to copper and mercury, their xerophytic existence, and the high water content of their wind-disseminated conidia. Germ tubes of powdery mildews are commonly phototropic [Neger (168)], some reversibly so [Yarwood (236)], and light may greatly increase the percentage germination at night [Yarwood (238)]. Powdery mildews are sensitive to contact stimuli in the formation of their appressoria [Corner (45)]. Dickinson (60) found that different substrate membranes affected the direction of growth of germ tubes of mildew spores. *Erysiphe polygoni* shows a diurnal cycle of conidial germinability [Yarwood (238)], and *E. graminis* exhibits a diurnal response to nutrients [Yarwood & Cohen (251)].

The greatest increases in germ tube length of powdery mildews have resulted from the additions of chemicals to the substrate and many of these treatments also increased percentage germination. The addition of sucrose increased germ tube length [Graf-Marin (93)], and increased the percentage germination of spores produced on senescent leaves [Yarwood (235)]. Fructose [Yarwood & Cohen (251)] caused a greater increase in germ tube length than other sugars tested. Other chemicals which have increased germ tube length or percentage germination or both are: copper sulfate, ferric sulfate, potassium permanganate, glutathione, alpha tocopherol acetate, Tween 60 (polyoxyalkalene derivative of sorbitan monostearate), animal lecithin, liver extract, blood, milk, alkaline extracts of dried barley leaves, and alkaline extracts of chicken egg yolk [Yarwood (239, 241, 242, 244)]. While these are arranged in approximately increasing order of response, the materials were not simultaneously compared, and it is perhaps safe to say that none were tested under conditions for optimum response. Usually the separate effects of these chemicals were not additive in combination. For example, none of the many chemicals which produced increased growth when added to sucrose agar, produced any increase when added to sucrose yolk agar or fructose lecithin agar. Conidia of *Erysiphe graminis* on plain agar produced germ

tubes up to about 140μ in length, while conidia on sucrose yolk agar produced germ tubes up to about 700μ. This was a greater relative response to nutrients than shown by eight other powdery mildews, but the actual length of the germ tubes was greater for the conidia of *Sphaerotheca lanestris* than for any other powdery mildew tested.

The most plausible report of success in the culture of a powdery mildew has been by Hammarlund (99). His report of formation of conidia of *Sphaerotheca mors-uvae* from the pannose mycelium after the mycelium was detached from the host is not so surprising. This might be the result of extensive food storage in this specialized mycelium and can hardly be considered axenic culture or a useful lead to axenic culture. His report of producing conidia of *Erysiphe polygoni* (*E. communis*) in cultures starting with conidia or ascospores seeded in host (*Hypericum perforatum*) cell sap is more spectacular, even though he states he could not germinate the conidia produced in the latter cultures. This reviewer is inclined to depreciate reports such as this where the writer gives preliminary information of an important discovery and promises details in a later paper which in the subsequent 30 years apparently have not appeared. Promising initial results in the culture of *Oidium heveae* on artificial media are indicated by Young (253).

Among the Perisporiaceae, closely related to the Erysiphaceae, and with haustoria [Doidge (63)], apparently none has been cultured.

Uredinales.—Rusts are among the most highly specialized fungi known, and trials of axenic culture with them have probably been more extensive than with any other obligate parasites [Chester (44)]. Reports of attempted culture are not reviewed here unless specific information is indicated. Germination of pycnospores has been studied by Carleton (42), Plowright (182), and others. Sugar and honey are the only materials known to stimulate pycnospore germination. Our concept of pycnospores as male spermatia would suggest that these cells would be the least promising spores with which to study axenic culture.

The rapid initial growth of germ tubes from uredospores in water, and the cessation of growth in less than 24 hr. and before one mm. of growth is attained are best described by Stock (213). Slight increases in germ tube length due to nutrients have been reported by Carleton (41), Ezekiel (74), Mains (146), Wilhelm (228), and Yarwood (244). The several-fold increase in germ tube length due to self stimulation as reported by Ezekiel (74) and the increase in percentage germination due to vaseline or paraffin oil as observed by Melhus & Durrell (156) and Bailey (14) are greater than any response of rust spores to added nutrients. As further evidence of the self-stimulation phenomenon, Ezekiel found that anastomosis of adjacent sporelings resulted in longer germ tubes. Yarwood (244) used lightly seeded agar plates to avoid the self-stimulation effect and secured increased germ tube length from potassium permanganate, disodium alpha tocopherol phosphate, and glutamic acid. The effect of these chemicals, however, was

perhaps no greater than from adding more spores. The maximum length of germ tubes from uredospores in axenic culture is about 1,500μ as observed by Straib (214).

There is little reason to believe that pH is critical in axenic cultures of rusts, as Aronescu-Savulescu (10) found that uredospores of *Puccinia antirrhini* would germinate between pH 3.6 and pH 12.4. At the ends of germ tubes of uredospores, secondary uredospores have been observed by Sappin-Trouffy (194) and Spaulding (208). The germination of teliospores usually gives rise to promycelia with basidiospores, but "indefinite growth" of the promycelium is reported by Bailey (14), and formation of spherical bodies is recorded by Carleton (41). The much poorer germ tube growth from basidiospores than from uredospores [Reed & Crabill (188), Sappin-Trouffy (194)] is probably related to the small size and low food reserves of the basidiospores. The report by Ray (187) of the culture of a rust on an agar substrate is not accepted. The role of possible nitrogen fixation by rusts (D'Oliveira (64)] has not been appraised.

All other recent studies of the culture of rusts and other obligate parasites are dwarfed by the report of the axenic culture of *Gymnosporangium* by Hotson & Cutter (56, 116). These investigators first grew *Gymnosporangium*-infected cedar galls in tissue culture. After several months they observed a fungus growing on the agar substrate away from the host tissue. When this fungus, apart from the gall tissue, was transferred to standard media such as potato dextrose agar, the fungus continued to grow as a reddish mass. Inoculations on *Crataegus* were successful. This axenic culture of a rust has been questioned by Allen (1) and Savile (195) and should be confirmed by other investigators.

Fungi culturable in certain stages.—In the Peronosporales, Mucorales, Helotiales, and Agaricales, the perfect and imperfect stages may usually both be formed in culture. In the Taphrinales and Ustilaginales, the imperfect stage is usually formed in culture, but the perfect stage is not. This suggests that the imperfect stage is facultatively saprophytic and the perfect stage is more nearly obligately parasitic. In the Hypocreales and Sphaeriales, however, in nature the imperfect stage is usually parasitic and the perfect stage is saprophytic. Here we have the anomaly that the stage which is parasitic in nature is more easily maintained in axenic culture. This may be partly a matter of unfortunate terminology. The perithecial stage of *Claviceps*, for example, forms independently of the grass host, but may be considered parasitic on the *Claviceps* sclerotia, in the same way that shoots, roots, flowers, and embryos of higher plants may be considered parasitic on other parts of the same plants [Bastin (18) and Bessey (27)].

Clearly established specialized sexual spores of nonobligate parasites usually can not be germinated on nonliving substrata, and will not produce infection. For example, the spermatia of *Mycosphaerella brassicicola*, long considered pycnidiospores, do not germinate or cause infection [Snyder (207)], whereas the ascospores germinate readily, give rise to axenic cultures,

and cause infection. The failure of germination of spores of many other non-obligate parasites such as *Phymatotrichum omnivorum* [Roberts (191)] can not be as readily explained.

Miscellaneous fungi.—Many of the mycorrhizal fungi have neither been identified nor grown in axenic culture [Melin (158), and Modess (161)]. Of those which have been cultured, many showed a marked response to unidentified growth-promoting substances. The status of the root-invading *Rhizophagus* is uncertain. Butler (39), Magrou (144) and many others have failed to culture it, but Barrett (16) reported it in culture on hemp seed agar. Recently it seems to be inferred that *Rhizophagus* is really a *Pythium* [Harrison (102)].

The lichen fungi are commonly regarded as obligate parasites [DeBary & Gurnsey (58) and Caullery (43)], but at least a few have been reported in culture [Fink (76), Möller (162)].

The obligate or near obligate parasitism of several fungi living on insects [DeBary & Gurnsey (58) and Shanor (200)] is beyond the scope of this review, but some have recently been grown in axenic culture [Wolf (233)].

Darluca filum, a common parasite of rusts, is regarded as an obligate parasite by Chester (45), but Keener (124) reports culturing without difficulty strains of *Darluca* from several rusts.

Other miscellaneous fungi which have resisted axenic culture are *Cercospora koepkei* [Dignadice & Orillo (62)], *Elytroderma deformans* [Lightle (135)], *Macroderma Curtisii* [Luttrell (141)], *Phyllachora punctatum* [Orton (174)], and *Cyandothea tripolii* [Wolf (231), Butler & Jones (40)].

Mitochondria.—Mitochondria are commonly regarded as integral parts of cells [Newcomer (169)] but Portier (181) and Wallin (222) considered them microorganisms, and reported that they had cultured them.

Nematodes.—Some nematodes are parasitic on higher plants or on the microflora which inhabit their digestive tracts or on both. No nematode parasitic on plants has been grown in axenic culture [Goodey (91)], but Mountain (165) has grown them on root tissue cultures apart from other microorganisms. Dougherty et al. (66, 68, 69) have maintained the free-living nematode *Rhabditus briggsae* in axenic culture, and their methods might be expected to be successful with other species.

Higher plants.—About 1700 species of spermatophytes in 12 families are parasitic on other spermatophytes [MacDougal & Cannon (143), Melhus & Kent (157)]. A continuous gradation from non-parasitic to parasitic forms is known to exist and parasitic members are believed to have evolved from non-parasitic members in comparatively recent times [Peirce (177)]. Many forms, including the dodders, mistletoes, and broomrapes, are commonly believed to be obligate parasites, but at least two dodders have been grown in axenic culture [Loo (137), Molliard (163)].

XUM

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BIOLOGICAL RELATIONS OF PLANT VIRUSES[1]

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Evidence from the study of more than 200 plant viruses indicates that a wide range of diversity of relationships has developed between viruses and the plants in which they cause disease.

Relationships with insects, which are responsible for transmission of most plant viruses so far as known, may vary from a simple mechanical association in which virus is carried from plant to plant on the insect's mouthparts to a more complex biological relationship involving increase of the virus in the insect and even to the passage of the virus to the succeeding generation through the egg stage. The literature dealing with the relationships of plant viruses to their insect vectors has been reviewed recently by Black (1) and will receive no further attention in this summary. The discovery that mites may transmit viruses is so recent that information is not available as to modes of transmission by these agents.

The earlier literature dealing with invasion of plants by viruses and the relation of viruses to different types of tissues was reviewed by Bennett (2). More recently, Esau (3) summarized the information on cytological and anatomical effects of viruses on plants, and Crafts (4) reviewed the literature dealing with virus movement.

Information on how viruses enter plants, occupy cells and tissues, move from points of introduction, the paths followed, and how they produce their typical reactions and other phenomena in the interplay between host plant and virus is far from complete. Slowly, however, more evidence is becoming available from which it will be possible to formulate more accurate concepts. It is the objective of this review to summarize available information on the relationships of viruses to the plants they invade and to suggest possible interpretations of some of the indicated relationships which may be clarified more fully with additional information.

Modes of Entrance of Viruses into Plants

Plants may be infected with viruses by one or more of a number of methods of inoculation. There is evidence, however, that viruses are unable to infect plants by entry through natural openings or through unbroken epidermis of leaves or other plant parts. Duggar & Johnson (5) reported infection of tobacco plants with tobacco mosaic virus through stomata of leaves. However, Sheffield (6) was unable to verify this report and suggested that the infection obtained did not take place through the stomata but resulted from accidental infection through wounds made in the process of inoc-

[1] The survey of the literature pertaining to this review was concluded in October, 1955.

ulation. Caldwell (101) introduced tobacco mosaic virus into the intercellular spaces of tobacco leaves by subjecting the leaves to reduced pressure in a virus suspension, but found no evidence that infection resulted if cell walls were not broken. Diachun, Valleau & Johnson (7) found that tobacco mosaic virus entered intercellular spaces of leaves readily through stomata of water-soaked areas but that infection did not result from such entry.

Although plants have a high degree of protection against entrance of viruses through the epidermis and natural openings such as stomata, hydathodes, and lenticels, they are extremely vulnerable to infection through various types of injuries produced by insects, by man, and by other agencies. Methods of infection of plants by viruses include entrance of virus through injuries of various types, entrance through tissue union, introduction by means of parasitic plants, and introduction through insect feeding. The effectiveness of these different modes of transmission of viruses varies with the virus and with the plants involved. All, however, are useful in the experimental transmission of viruses and all, except transmission by parasitic plants, are important in the natural spread of virus diseases in economic plants.

Transmission by juice inoculation.—One of the common methods of introducing viruses into plants experimentally is by rubbing virus suspensions over leaf surfaces. Many of the mosaic-producing viruses are transmissible by this procedure. Rawlins & Tompkins (8) showed that the addition of a small amount of carborundum to the leaf or to the inoculum increased the the amount of infection from rubbing. They suggested that the carborundum crystals pierced leaf cells and allowed virus particles to enter cells that are not injured sufficiently to prevent virus multiplications. This concept of virus entry is supported by results of tests by Beraha, Varzandeh & Thornberry (9), who found that tobacco mosaic virus is not adsorbed on carborundum particles at pH 8.5, the optimal pH for infection, which indicates that the abrasive particles do not carry the virus into the tissues but only produce a type of wound suitable for entry.

Many plant viruses either are not juice-transmissible or are transmitted in this way only with great difficulty. The rubbing method of inoculation is probably effective in introducing any virus into living plant cells. Failure to produce infection by juice inoculation, therefore, must result from inability of the virus to multiply and move from the inoculated cells. This failure, in some instances, appears to result from the action of inhibitors either in the inoculum or in the inoculated cells. In other instances, plants apparently are immune from infection by mechanical rubbing because the method of inoculation results in the introduction of the virus only into cells in which it is normally unable to increase.

Transmission by tissue union.—It is characteristic of plant viruses that they are graft-transmissible in the plants in which they produce systemic invasion. Even those viruses that are not fully systemic usually are graft-transmissible if the tissue selected as inoculum is invaded by the virus and

becomes established on the inoculated plant. Graft transmissibility of a disease-inciting agent, in the absence of evidence of fungi or bacteria as etiological factors, constitutes one of the decisive tests in determining whether an abnormal plant condition is caused by a virus. In fact, several diseases of plants, for which no natural agent of transmission has been found and which are not transmissible by other conventional methods of inoculation, have been classified as virus diseases on the basis of graft transmission alone.

Graft transmission of viruses is easily accomplished. In general, any tissue that contains virus and which will form a union with a healthy plant will serve as effective inoculum. Infection may sometimes occur even when the tissue serving as the inoculum survives for only a short time; however, some union appears to be required for transmission if the virus is not of a type transmissible by rubbing.

The extent and duration of the union necessary for transmission vary with different viruses. In studies of contact periods required for the transmission of viruses causing yellow, little peach, rosette, and mosaic of peach by budding, Kunkel (10) found that the mosaic virus usually passed the graft union in two to three days but the other viruses required from eight to fourteen days.

Bennett (11) found that when scions of Turkish tobacco infected with the viruses of tobacco ringspot, cucumber mosaic, and curly top were grafted to healthy tobacco plants, no infection occurred during the first day of contact. Both the ringspot virus and cucumber mosaic virus began to move into the stock the second day but the ringspot virus moved more rapidly. Curly top virus did not begin to move through the union until the fifth day. It was suggested that the viruses of ringspot and cucumber mosaic were able to move through the union as soon as a meristematic or early parenchymatous union was established between stock and scion, whereas passage of curly top virus was delayed until a union of phloem tissue was well advanced or completed. It seems probable that the cells in which viruses are able to move may be the determining factor in the speed with which viruses move through graft unions; mosaic viruses that occur in the parenchyma move quickly, whereas in other types that may have greater tissue restrictions movement is delayed.

Transmission by tissue union is undoubtedly one of the chief methods of dissemination of a number of viruses. Bitancourt & Fawcett (12) presented statistical evidence indicating that citrus psorosis may be transmitted by natural root grafts in the citrus groves of California. Thomas & Baker (13) found many natural root grafts between healthy and diseased carnation plants in association with the spread of carnation mosiac in the absence of other known methods of spread. This finding is further evidence that transmission through root grafts may occasionally be a means of spread of some viruses under natural conditions.

However, most transmission of viruses by tissue union results from

grafting and budding in the ordinary processes of vegetative propagation. Viruses of fruit and ornamental trees and shrubs are spread extensively by this means.

Some of the unexpected and inconsistent results from grafting, attributed to incompatibility between stock and scion, are now known to be the result of transmission of unsuspected viruses. For many years, for instance, sour orange was considered to be incompatible as a rootstock for sweet orange in South Africa but completely compatible as a stock for sweet orange in other parts of the world. This difference proved to be due to the presence in South Africa of citrus tristeza virus which caused death of sweet orange trees on sour orange rootstocks but which caused no recognizable injury to sweet orange trees on other rootstocks such as sweet orange or rough lemon. It is probable that other viruses, not yet recognized, are transmitted in a similar manner.

Transmission by means of parasitic plants.—Evidence that a virus can be transmitted by a species of dodder, *Cuscuta subinclusa*, was reported first in 1940 (14), and it has been found since that many viruses are transmissible by this type of parasitic plant. Transmission of virus by dodder under natural conditions is of little or no importance, but the method of transmission has proved of considerable value in certain phases of virus research. Since, in general, species of dodder have wide host ranges, they are especially useful in transmission tests between plant species that are widely separated botanically, and particularly with those viruses for which no other means of transmission are available because of vector limitations or other factors.

Kunkel (15, 16, 17) has utilized *C. campestris* extensively in the transfer of viruses to new host plants. These transfers have included, among others, transmission of the virus of cranberry false blossom to periwinkle, tomato, and other plants; potato witches'-broom virus from potato to a number of plants not previously known to be susceptible; and more recently he succeeded in transferring the virus of alfalfa witches'-broom virus to carrot, tomato, and periwinkle. These extensions of the potential host range of the respective viruses have not been feasible by other means of inoculation.

By taking advantage of the selective capacity of *C. campestris* to transmit viruses, Johnson (18) discovered that the disease previously known as white clover mosaic is caused by two distinct viruses, only one of which is transmissible by the species of dodder used. Giddings (19) found that the beet leafhopper acquires curly top virus more readily by feeding on dodder growing on an infected tomato plant than by feeding on the tomato plant itself. Dodder plants may be found useful also in the study of the transport of viruses, carbohydrates, and other material. Since the different species of dodder, through their haustoria, make direct connection with the phloem and xylem of their host plants, they would appear to be particularly well adapted for use in the study of unidirectional movements that occur in the vascular system of plant stems.

The probable mechanics of transmission of viruses by species of *Cuscuta*

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has been discussed by Bennett (20). Anatomical studies by Peirce (21) and by Thoday (22) indicate that species of dodder make very close unions with their host plants. The xylem elements of the host and parasite unite directly. The sieve tubes of host and parasite are connected by a footlike extension of the parasite which attaches to the sieve tubes of the host. The relationship, therefore, between dodder and its host is essentially that of one plant grafted to a second, but with the species which serves as the stock, supplying all of the nutritional requirements of the scion species.

Considering the type of relationship that exists between species of dodder and their host plants and the evidence that virus movement in the phloem of infected plants is correlated with the transport of photosynthates, it would be expected that virus present in the phloem tissue of the host would move readily into the parasite. Transmission to a second plant would depend on the ability of the virus to move through haustoria of the parasite into healthy plant tissue before it becomes inactivated in the tissue of dodder.

Viruses that produce mottling or similar disturbances in the parenchyma, and that are transmissible by dodder, apparently fall into one of two classes and are either transmitted to a very high or to a very low percentage of inoculated plants. If dodder is a host of the virus, as in the case of the viruses of cucumber mosaic and dodder latent mosaic, a high percentage, or all, of the inoculated plants becomes infected; if dodder is not a host, little or no infection is obtained.

Apparently most of the viruses that have been tested thus far and that produce yellows- or leaf-curl diseases are transmissible by dodder. Dodder has not been found to be a host of any of these viruses and usually they are transmitted only to a low percentage of inoculated plants.

It is possible that these results may have an explanation, in part at least, in the facility with which different viruses are able to invade specific tissues. Viruses able to invade the parenchymatous cells of the haustorium may move readily from the outer cells of the haustorium into the adjacent cells of the host through perforations that appear to be present in walls of the cells of both dodder and its host (20). This avenue of invasion, however, would not be so readily available to those viruses, like curly top virus, that are apparently able to invade parenchyma only to a limited degree, but which are transmissible by means of dodder. It seems probable that transmission of such viruses may be effected by temporary and limited reversals of normal directional movements of materials in the phloem of the parasite. These reversals would be sufficient to carry virus the short distances required for entrance into the phloem of the host plant. This concept is supported by results of tests by Cochran (23) in which it was shown that shading or defoliation of dodder-inoculated plants resulted in a marked increase in infection with tobacco mosaic virus.

Certain anomalies in the transmission of viruses by dodder have been pointed out by Kunkel (17). For instance, alfalfa witches'-broom virus was

transmitted from alfalfa by *C. campestris* to carrot, tomato, and periwinkle, but not to alfalfa or parsley. Peach rosette virus and peach X-disease virus were transmitted from peach to carrot, tomato, and periwinkle, but not from any of these plants to peach. Aster yellows virus was transmitted readily to red clover by *C. campestris* but with difficulty to China aster, whereas the natural vector, the aster leafhopper, transmitted the virus readily to China aster but rarely to red clover.

No completely satisfactory explanation for the differences in ability of *C. campestris* to transmit viruses to different host plants is available on the basis of known facts. However, all of the viruses involved are of a type that may have a high degree of restriction to the phloem. Therefore, transmission by dodder may be dependent on virus movement out of haustoria into the healthy plant in a direction counter to the normal transport of food materials, during temporary reversals of food flow between parasite and host. Obviously, if infection is dependent on virus entry correlated with food transport, host plants able to maintain a continuous flow of food materials into the parasite would be less likely to become infected than those that have intermittent food deficits in the phloem.

Introduction by insects.—Most insects that transmit plant viruses have mouthparts that enable them to pierce plant tissues without causing serious injury to individual cells. A few viruses are transmitted by insects of other types. Spotted wilt virus is disseminated by thrips. These insects have rasping mouthparts and introduce virus into ruptured cells, possibly through the agency of salivary deposits that are left in the injured cells in the feeding process. Certain other viruses are transmitted by chewing insects. Turnip yellow mosaic is transmitted in nature by the turnip flea beetle. Markham & Smith (24) showed that this insect is an efficient vector of the virus because it regurgitates from the foregut while feeding and brings virus, previously swallowed, into contact with newly injured cells.

Insects with piercing mouthparts may feed on many kinds of tissues, including phloem, various types of parenchyma, and even woody tissue. The exact method whereby these insects introduce virus into the plant is not clearly defined but it is known that insects introduce appreciable quantities of salivary secretions into the cells in which they feed. These secretions solidify quickly, frequently as a sheath around the mouthparts, and remain in the cells after feeding is discontinued. Storey (25) expressed doubt that a medium that solidified so quickly is responsible for carrying virus into the plant. However, Smith (26) found that after beet leafhoppers carrying curly top virus had fed on drops of water through a membrane, other leafhoppers could acquire virus by feeding on the same liquid. It seems certain, therefore, that the curly top virus is introduced into liquid media and into plants along with the salivary deposits of the leafhopper vector. It seems probable that, in general, viruses are introduced into the plant by this type of vector through the medium of the salivary secretions. However, it is possible that some of the viruses transmitted by insects in short feeding periods may enter the cell

as simple contaminants on the stylets of the vector and may not necessarily be associated with salivary deposits under all conditions.

Mosaic and ringspot viruses usually are effectively introduced into plants by aphids in very short feeding periods. Sylvester (27) found that plants are infected with the *Brassica nigra* mosaic virus by the green peach aphid, *Myzus persicae*, in a feeding period of five seconds. Similar short feeding periods by aphids were found by Hamlyn (28) to be sufficient for transmission of cabbage black ringspot virus. Bradley (29) found that *M. persicae* transmitted henbane mosaic virus in feeding periods of 5 to 10 sec. The probability that a single puncture would result in infection reached a maximum in 20 to 30 sec., during which time the stylets of the vector did not penetrate to the center of the punctured epidermal cell and very little saliva appeared to be injected. In general, short feeding periods by vectors appear to be effective in the transmission of viruses that produce diseases characterized by various types of mottling or leaf spotting. It seems probable that these viruses need only to be introduced into epidermal or other types of parenchymatous cells to become established sufficiently to produce infection.

Longer feeding periods and deeper penetration of tissues appear to be more effective in transmission of viruses that cause leaf curling, stunting, and yellowing, in the absence of spotting. Watson (30) found that the aphid, *M. persicae*, requires a minimum feeding period of about seven min. to transmit sugar beet yellows virus and that longer feeding periods gave more infection. This virus is transmitted with considerable difficulty by juice inoculation and it seems probable that introduction of the virus into any of the various types of leaf parenchyma cells usually results in little or no infection. MacCarthy (31) found that the green peach aphid requires a minimum feeding period of 30 min. to infect plants with potato leafroll virus. This and other evidence indicates that infection with this type of virus usually is produced most readily when the virus is introduced into the phloem region of the vascular bundle. Infection with some of these viruses apparently is produced only when virus is introduced into phloem tissue.

Houston, Esau & Hewitt (32) found that when the feeding of a natural leafhopper vector of the virus of Pierce's disease in grape was restricted to the cortex of the stem no infection resulted. Infection was produced only when the insects fed on the woody cylinder. In this case, apparently the virus must be introduced into the xylem in order for it to become established in the plant.

It seems probable that most of the different types of sucking insects that are vectors of plant viruses introduce virus into all of the types of tissue in which they feed. Evidence from the prevalence and location of salivary deposits in tissues would support this view. It would seem, therefore, that results in terms of infection, following different types of feeding by insects, may provide useful information with respect to relative degrees of resistance of specific plant tissues to virus infection.

Tissues Invaded by Viruses

In contrast to pathogenic bacteria and fungi which usually produce local effects, viruses invade plants extensively and usually may be considered systemic pathogenic agents. However, viruses are not always systemic in that they do not invariably invade all of the living cells of infected plants. The extent of invasion varies with the virus and with the plant involved. A few viruses appear to have a high degree of restriction to specific plant parts or organs. Hutchins (33) and Hutchins *et al.* (34) have shown that peach phony disease virus is more concentrated in the root system than in the tops of diseased trees and that it appears to be limited to the root system in some trees. Smith (35) found that the virus of tobacco necrosis usually is confined to the root system of affected plants and that it does not become fully systemic even when inoculated into the tops.

Usually, however, viruses invade roots, stems, leaves, flowers, and fruits of plants more or less indiscriminately. Differences noted in selective invasion of plants by viruses usually have been associated with specific tissues. Even the partial or complete restriction of viruses to roots may be more nearly associated with specific tissues than with the parts involved. More and more evidence is accumulating that indicates a high degree of variation in the susceptibility of different tissues to infection and to injury by different viruses. These variations are associated, to an appreciable degree, with the fundamental plant tissues such as meristem, parenchyma, phloem, and xylem.

Meristem.—It is difficult to determine directly the extent to which viruses invade meristematic tissue of the growing points and cambial regions of infected plants because of the small volumes of meristem available for test and the uncertainty of obtaining meristem free of contamination from adjacent tissues. The suppression of chlorophyll production by many viruses of the mosaic-producing type suggests that invasion of the growing points may occur in the early stages of cell maturation. There is little evidence of direct injury to individual cells of meristematic tissue. However, this may be due to a time factor or to natural resistance to injury rather than to delayed invasion. Cambial cells appear to remain uninjured in most virus-infected plants. Growing points are rarely killed by viruses and, in those cases where death does result from virus infection, it has not been determined whether the initial lethal effects are produced primarily in the meristem or in the adjoining, more mature tissue. Often, severe injury to terminal parts is followed by a high degree of recovery and production of new growth, but it is not known whether this increased tolerance of the virus develops in the terminal meristem or in partially differentiated cells.

In an attempt to obtain direct evidence on the occurrence of virus in the primordial meristem of tomato plants infected with aucuba mosaic virus, Sheffield (36) dissected out growing points from stem tips and roots and tested for virus. She concluded that this virus invades the meristems of both the shoots and roots of this plant.

Lackey (37) reported recovery of curly top virus from meristematic

tissue of root tips of sugar beet and from the cambium of tobacco stems. Much evidence indicates that the curly top virus (38) occurs in very low concentrations, or not at all, in the parenchyma of these plants. Occurrence, therefore, of curly top virus in the meristem of infected plants in the indicated concentrations must be followed by a high degree of inactivation of the virus in those cells that matured into parenchyma.

Holmes (39) found that dahlia clones, infected with spotted wilt virus, may be freed of virus by removing the extreme tips of infected shoots and allowing these to root and develop into new plants. He (40) found also that clones of sweet potato infected with the virus of internal cork disease were freed of virus by this type of propagation. In these instances, the meristem in infected plants appeared to be free of virus.

In summary, it may be stated that available evidence indicates that certain viruses may be able to invade primary meristematic tissue. At present there is little evidence that serious injury results from such invasion. In some instances, growing points of affected plants appear to be free of virus, which would indicate that in these cases the meristematic tissue may have a high degree of resistance to virus invasion.

Parenchyma.—Parenchyma tissues, in their reaction to virus invasion, show a wide range of response from essential immunity to extreme susceptibility. All types of parenchyma tissues appear to be unfavorable for invasion and increase of curly top virus (38), and this may be true to a greater or lesser degree with other viruses that give evidence of causing primary disturbances in vascular tissues.

Various assay methods indicate that those viruses that cause primary injury in parenchyma occur in highest concentrations in this tissue. Some viruses may be largely restricted to parenchyma tissue in certain of their host plants or under certain environmental conditions.

Tobacco mosaic virus produces only primary lesions on inoculated leaves of sugar beet, on some varieties of bean, and often on *Nicotiana glutinosa*. Age of tissue, degree of sensitivity of invaded tissue to action of the virus, environmental conditions, and type of parenchyma all appear to be factors which may influence degree of restriction of viruses to parenchymatous tissues.

Some strains of cucumber mosaic virus (2) produce only primary lesions when inoculated into mature beet leaves, but systemic infection results when aphids introduce the virus into the plant, presumably into very young leaves. This virus usually does not cause necrosis in mature beet leaves and continues to spread slowly through the parenchymatous tissues of the leaf so long as the leaf remains alive. Broadbent (41) found that the cabbage black ring virus occurs mainly in the older leaves and is localized in parts that show symptoms. Restriction of potato virus X to parenchyma in certain varieties of potato was found by Corbett (42) to be dependent in part on the age of the leaf. Older leaves produced only primary lesions, whereas inoculation of younger leaves was followed by systemic infection.

Cockerham (43) found that potato mosaic viruses A and X are limited to local areas in certain varieties of potato due to rapid killing of infected cells which prevented further spread of virus. This discovery is reported to have made possible the elimination of these viruses from potato varieties possessing genic factors for high sensitivity to injury.

Apparently different types of parenchyma vary in their susceptibility to invasion and in their ability to support virus increase. Hutchins (33) and Hutchins *et al.* (34) presented evidence indicating a high degree of restriction, or complete restriction, of the peach phony virus to the woody cylinder of infected plants, which suggests a restriction of the virus to xylem parenchyma. No evidence is available that indicates that this virus invades parenchyma in the cortex, leaves, or fruits.

Even different types of leaf parenchyma may vary in the concentrations of virus they contain and possibly also in their ability to support virus increase. It was shown at the Rothamsted Experimental Station (44) that irradiation of virus-infected leaves with ultra-violet light resulted in inactivation of virus in the epidermal cells but produced little effect on virus in cells more deeply situated. By taking advantage of this differential inactivation, Bawden, Hamlyn & Watson (45) found that exposing both surfaces of leaves systemically infected with cabbage black ringspot virus or henbane mosaic virus to ultra-violet radiation decreased the infectivity of expressed sap to about one-fifth that of untreated leaves. As irradiation probably inactivated virus mainly in the epidermis, which occupied about one-fourth of the volume of the leaves, these viruses seemed to occur in much higher concentration in the cells of the epidermis than in the cells of the mesophyll. This was not found to be true of leaves infected with tobacco mosaic virus, which led to the conclusion that viruses of different types can occur in different relative concentrations in different parts of the leaf.

Thus far, there is no convincing evidence that a virus may be restricted to parenchyma tissue in all of its host plants under all conditions. Indeed, it would seem rather improbable that such a virus could be perpetuated indefinitely except possibly under very unusual circumstances. However, there appear to be numerous instances of restriction of virus invasion to parenchyma in specific plants or under certain environmental or nutritional conditions.

Xylem.—The xylem tissue of plants is composed largely of nonliving tracheal and wood cells and different types of living parenchyma cells which may have very different relationships to viruses.

The tracheal elements are of interest chiefly because they provide for rapid and extensive transport of water to all parts of the plant. Plants may be induced to take up relatively large quantities of liquids through the tracheae in cut ends of petioles or stems. It would appear, therefore, that one of the simplest methods of inoculating plants with viruses would be to introduce them into the plant through the tracheal elements of the vascular system. Undoubtedly this method of introducing viruses into plants has been

tested with a large number of viruses, but thus far, evidence indicates that infection rarely results from this method of inoculation.

Caldwell (46) showed that tobacco aucuba mosaic virus was taken up readily by tomato plants through cut ends of petioles but that no infection resulted unless the plants were injured in such a way as to release virus into adjacent living cells. Caldwell (47) found also that water produced by guttation from the hydathodes of tomato plants infected with tobacco mosaic virus was free of virus. This evidence indicates that tobacco mosaic virus cannot pass through walls of tracheal cells in either direction. This is in agreement with other evidence indicating that the cellulose structure of cell walls in general may be impermeable to viruses.

However, it should be noted that the earlier tests of permeability of cell walls to viruses were made with tobacco mosaic virus which is a relatively long rod. It is possible that smaller viruses, particularly the smaller spherical viruses, may be able to pass through walls that would prevent passage of tobacco mosaic virus. In fact, some of the results of more recent work with the virus of Pierce's disease of grape have raised the question anew as to whether tracheal walls may not permit passage of certain types of viruses.

Houston, Esau & Hewitt (32) showed that leafhopper vectors of the virus of Pierce's disease of grape fed by preference in the xylem and that they did not produce infection when feeding was restricted to the cortex. It was demonstrated rather conclusively that the virus may occur in the tracheae. Esau (3) expressed the view, however, that increase occurs in the xylem parenchyma. If increase is restricted to the xylem parenchyma, the limited evidence of travel in the tracheae and the absence of evidence of ability of the virus to move in the phloem indicate that this virus may be able to pass through tracheal walls in either direction. However, there appears to be no evidence that demonstrates conclusively that this virus does not increase in the tracheae. Apparently it has not been determined whether infection can be produced by introducing suspensions of the virus directly into the vascular system. Increase in the tracheae would involve increase in a nonliving medium. This has not been found to occur with any plant virus but there would seem to be no reason to assume that viruses capable of increasing in a nonliving medium do not exist.

Very little is known regarding the relationships of the majority of plant viruses to living parenchyma of the xylem beyond the fact that certain of the mosaic viruses are able to bridge rings in stems by passing through living wood tissue and that certain of the yellows-producing types of viruses appear unable to move through living cells of the woody cylinder of stems. It would seem probable, on the basis of available information, that most viruses that produce mosaic may invade living cells of the xylem to a greater or lesser degree. However, the peach phony disease virus appears not only to be able to invade the xylem, but also to have a high degree of restriction to this tissue.

Hutchins (33) obtained infection with the phony disease virus only when

woody tissue of diseased roots was grafted to similar tissue of healthy plants. Hutchins *et al.* (34) later succeeded in transmitting the virus from tops of diseased trees. However, it was not transmissible by bark grafts. It was concluded that the virus is probably restricted to the woody cylinder in both tops and roots and that it probably multiplies in the wood parenchyma and medullary ray cells. It was suggested that the apparently higher concentration of virus found in the roots is attributable to the presence of a proportionally higher volume of living cells in the woody cylinder of that part of the plant.

Phloem.—It is probable that nearly all viruses are able to enter phloem tissue and to utilize phloem strands in their invasion of different plant parts. Apparently some viruses travel in the phloem without causing obvious disturbances; others produce serious upsets in the anatomy and functioning of phloem which may extend to the vascular bundle as a whole and into adjacent tissue.

Viruses that produce mosaics apparently cause little or no direct injury in the phloem. Some of these viruses have difficulty in entering the phloem and it may be that, in some instances at least, those viruses that reach their highest concentrations in the parenchyma may be able to increase in the phloem only to a limited extent or not at all. Tobacco mosaic virus (48, 49, 50) has been shown to be able to pass through long sections of tomato stems without producing infection in certain sections. Kunkel (49) pointed out that this observation suggests an inability of this virus to multiply in the sieve-tube cells.

The evidence available at the present time indicates that those viruses that produce leaf curls and general yellowing in the absence of mottling are closely associated with the phloem. Several viruses of this type are known to produce obvious injury in the vascular system, and symptoms usually are those that could be induced by direct action of the virus in the phloem. The literature dealing with the various types of pathological injuries to the phloem caused by viruses has been thoroughly reviewed by Esau (3). Marked disturbances in the phloem were caused only by viruses of the leaf curl and yellows types. The cytological and anatomical abnormalities described may result from direct action of virus on phloem cells.

Some of these viruses appear to have a high degree of restriction to the phloem. This is indicated by localization of primary symptoms in the vascular regions, by higher concentrations of virus in the phloem than in other types of tissue, and by results of ringing tests. Curly top virus (38), for instance, occurs in relatively high concentrations in the natural exudate from the petioles and blades of infected beet leaves and this exudate is apparently derived directly from the phloem. This virus failed to pass rings in stems of tobacco and *N. glauca* that severed phloem continuity but which did not destroy wood parenchyma and medullary rays in the ringed area. Similar evidence of a close association of the virus of raspberry leaf curl

(51) with the phloem was obtained with ringing experiments in which raspberry canes were used.

It seems probable that viruses that give indication of having a high degree of restriction to the phloem may multiply in this tissue. Whether the multiplication that occurs takes place in the phloem parenchyma, as suggested by Esau (3), or in the sieve tubes, or in both, has not been determined. It seems certain, however, from rates and directions of movement of viruses through the plant, that viruses present in the phloem are associated with those cells through which rapid movement of solutes occurs, which suggests the possibility of multiplication in the sieve tubes.

Seed Transmission of Viruses

Plants show a remarkable degree of freedom from transmission of viruses through seeds when it is considered that many of the viruses that infect plants appear to be able to invade all types of tissues and nearly all of the living cells of infected plants. Several viruses have been found to be seed transmitted to a greater or lesser degree but, in general, a protective mechanism has evolved in plants that apparently provides immunity to embryo infection by the great majority of the more than 200 known plant viruses.

Among the viruses reported to be seed transmitted are those causing bean mosaic (52), cucumber mosaic (53, 54), lettuce mosaic (55), cowpea mosaic (56), tobacco ringspot (57, 58), hops chlorotic disease (59), dodder latent mosaic (60), asparagus bean mosaic (61), elm mosaic (62), barley false stripe (63), Irish yellows (64) of sugar beet but not other types of beet yellows, squash mosaic (65), cherry ringspot (66, 67, 68), cherry yellows (66, 68), and an unnamed disease of sunflower in Argentina (69).

Even with those viruses that are seed transmissible there is a degree of protection against embryo infection that often is of a very high order, and there appears to be no instance reported of the transmission of a virus through all of the seeds of an infected plant. Often, the percentage of seeds through which a virus is transmitted is very low.

Newhall (55) found about 3 per cent seed transmission of lettuce mosaic virus, Bennett (60) reported 4.8 per cent transmission of dodder latent mosaic virus through seeds of *C. campestris*, and Middleton (65) found only from 0.14 to 0.96 per cent seed transmission of squash mosaic virus through the seeds from diseased squash plants. However, higher percentages of seed transmission are obtained with certain other viruses. The virus of bean mosaic is sometimes transmitted through relatively high percentages of seeds of diseased plants. Traversi (69) reported transmission of a virus in Argentina through 17 to 43 per cent of the seeds of infected sunflower plants, and Desjardins, Latterell & Mitchell (70) reported transmission of tobacco ringspot virus through as high as 78 per cent of the seeds of the Lincoln variety of soybean.

Transmission of one virus through the seeds of a plant appears to be no

indication that other viruses will be transmitted through the seeds of the same plant. For instance, tobacco ringspot virus is transmitted through seeds of tobacco but none of several other viruses that infect this plant is reported to be transmitted through tobacco seeds.

Some viruses are transmitted through the seeds of more than one species of plant. Tobacco ringspot virus is transmissible through the seeds of tobacco (58), petunia (71), and soybean (70). However, transmission through the seeds of one plant species does not insure transmission through seeds of other hosts. Dodder latent mosaic virus (60) is transmitted through the seeds of *C. campestris* but not through the seeds of cantaloupe, pokeweed, or buckwheat. Even varieties of the same species may vary. Clinch & Loughnane (64) reported seed transmission of the beet yellows virus only through seeds of beet "Family 41," and Couch (72) reported transmission of lettuce mosaic virus through a low percentage of seeds of the Bibb variety of lettuce but found no transmission through seeds of the variety Cheshunt Early Giant.

Some viruses are known to occur in seeds of infected plants in relatively high concentrations without being able to enter the embryo and produce infection in the young seedling. Allard (73) reported a number of years ago that the virus of tobacco mosaic occurs in both immature and mature seeds but the infected seeds do not produce infected seedlings. Ainsworth (74) found tobacco streak virus in the seedcoat of tomato seeds but not in the embryo. Bennett & Esau (75) found relatively high concentrations of curly top virus in the seedcoat and perisperm of beet seeds but the embryos were free of virus and always produced virus-free seedlings.

The factors and processes involved in transmission of viruses through seeds are largely undetermined. In most instances it is not known with certainty whether lack of seed transmission results from failure of virus to infect the embryo or whether infection occurs and the virus is inactivated later. Kausche (76) presented evidence interpreted as supporting the latter hypothesis. Cheo (108) showed that the virus of southern bean mosaic occurs in all of the flower and fruit parts of systemically infected bean plants, including the embryo. The virus was transmitted through immature seeds but not through mature seeds. Crude extracts from bean seeds were found to contain an inhibitor when added to southern bean mosaic virus. In this case the virus entered the embryo but was inactivated during the ripening process of the seed.

However, evidence indicates that certain other viruses may not be able to infect the embryo or even to enter and retain activity in generative cells. Viruses with a high degree of restriction to the phloem, as apparently is true for beet curly top virus and perhaps other viruses of this general type, would not be expected to be seed transmitted since there is no vascular connection between the embryo and the mother plant. There is some evidence that seed transmission may be restricted to those viruses that produce mottling

and other disturbances in the parenchyma and which are able to extensively invade a wide range of tissue types.

Much evidence has accumulated that points to the conclusion that seed transmission of some viruses may be determined at the time of the differentiation and maturation of ovules and pollen or during the development of the embryo following fertilization.

Nelson (77) states that seeds from plants infected after flowering rarely transmit the virus of bean mosaic, and Couch (72) found that lettuce plants inoculated with lettuce mosaic virus just before flowering produced fewer infected seeds than plants inoculated earlier. Plants infected after flowering had started did not transmit the virus through the seeds.

Reddick (78) found evidence indicating that the bean mosaic virus is transmitted through the pollen of bean. Nelson & Down (79) estimated from results of cross-pollination studies that 25 per cent of pollen grains and about the same number of ovules from infected plants carried bean mosaic virus.

Gold *et al.* (63) found rod-shaped particles in high concentrations in tissues of plants infected with barley false stripe. The particles were present in leaves, embryos, endosperm, pollen, unfertilized pistils, and seeds of healthy plants pollinated from infected plants, but not in seeds of healthy plants pollinated from healthy plants. Tests showed that about 10 per cent of the plants from seeds from healthy plants which had been pollinated from infected plants produced diseased seedlings. This evidence indicates clearly that the virus of barley false stripe occurs in pollen of infected plants and that it is transmissible through the pollen.

Evidence thus far indicates that those viruses that are seed transmissible are of a type likely to be found in high concentrations in parenchyma tissues. Also, where adequate tests have been made, seed-transmitted viruses occur in pollen of infected plants. There is little or no evidence that viruses that are not seed transmissible occur in pollen of infected plants but more extensive tests are needed before final conclusions may be reached on this point. It seems probable, therefore, that seed transmission may depend on the ability of a virus to enter the embryo sac directly or through the agency of the content of the pollen tube and infect the embryo in its early stages of of development. There is no evidence that the embryo may be infected during later stages of its development although, in some instances, the young seedling may become infected at germination time from virus contained in tissues surrounding the embryo.

Rates and Paths of Virus Movement and Factors Involved

The rate of invasion of plants by viruses is dependent in part on the type of virus and plant involved and in part on the tissue into which the virus is introduced initially. In the usual processes of inoculation, viruses may be introduced into superficial or more deeply situated parenchyma cells, di-

rectly into the phloem, or into the xylem elements. Increase of a virus usually begins soon after it enters the plant. Rate of increase probably varies with different viruses, and undoubtedly it is influenced by the host plant. The concentration of virus that is reached may, in turn, influence the rate of initial spread.

Studies of early stages of increase of some of the bacteriophages demonstrate a latent period following infection, in which no virus increase can be detected. Evidence as to the occurrence of a comparable latent period in the increase of plant viruses is still inconclusive. Uppal (80) demonstrated progressive increase in tobacco mosaic virus beginning 9 to 12 hr. after inoculation. Steere (81) found progressive increase in tobacco mosaic virus beginning about 20 hr. after inoculation. Yarwood (82) found no increase in the amount of tobacco mosaic virus recoverable during the first 5 to 7 hr., but after 8 hr. the amount of recovered virus increased at a logarithmic rate corresponding to a generation time of about 1 hr.

This evidence indicates that, by the methods used, tobacco mosaic virus is not recoverable in quantities indicating increase in the first few hours following inoculation. It may be, however, that failure to detect increase has resulted from lack of assay methods sufficiently refined to detect very low virus concentrations.

Some viruses are known to be able to move immediately after they are introduced into the plant. Curly top virus (38), for instance, may move as much as 6 in. in the first 6 min. after introduction into the plant, and it (83) may move out of beet cotyledons and produce symptoms in true leaves in 30 hr. Also, when this virus (20) was introduced into a stem of *C. subinclusa* by the feeding of viruliferous beet leafhoppers, it passed through 12 in. of stem and was picked up by nonviruliferous leafhoppers 3 hr. later. It seems probable from these results that movement, and probably increase, of the curly top virus may begin immediately after the virus is introduced into the plant. However, tests of rates of movement of viruses away from points of introduction are too limited to justify general conclusions.

Movement in parenchyma.—All available evidence indicates that movement of viruses through parenchyma tissue is a very slow process. The rate of invasion of parenchyma by a number of viruses may be estimated rather accurately by measuring the radial spread of primary lesions in inoculated leaves by use of the starch-retention technique described by Holmes (84), or by other means. Estimates of increase in size of lesions produced by viruses of tobacco mosaic, beet mosaic, dodder latent mosaic, and other viruses, indicate that movements up to 2 mm. per day may occur. However, movement of some viruses may be much slower under some conditions. Uppal (80) estimated that the virus of tobacco mosaic moved from the upper to the lower epidermis of leaves of *N. sylvestris* at the rate of 7 to 8 μ per hr. Kassanis (85) states that primary lesions resulting from inoculation of beet leaves with yellows virus did not appear in less than two weeks, and Costa & Bennett (86) found that about 20 days were required by this virus for the

production of primary lesions about 1 mm. in diameter. It is evident from these results that rates of virus movement in parenchyma may vary widely. Even the fastest rates observed, however, are slow and of an entirely different order from those that occur in the vascular system of the plant.

Studies by Livingston & Duggar (87) indicate that the virus of tobacco mosaic is much more concentrated in the cytoplasm than in the vacuoles of infected cells. Martin & McKinney (88) found comparatively little virus in vacuolar sap and concluded that the cytoplasm contains most of the virus present in living tissue. This evidence, although not conclusive, indicates that cytoplasm is the medium in which movement through parenchymatous cells takes place.

After a virus is introduced into the cytoplasm of a plant cell and increases, it seems probable that it is distributed throughout the cell by protoplasmic streaming, possibly aided by the processes of diffusion and increase. It soon moves from the inoculated cell into adjacent cells. In view of the evidence indicating that, in general, viruses are unable to penetrate the cellulose structure of the cell wall, the most plausible avenue of passage from one parenchyma cell to another, as pointed out by Sheffield (89) and by Martin & McKinney (88), is along interconnecting plasmodesmatal strands. Livingston (90) found plasmodesmata distributed throughout all of the living tissues of the tobacco plant and concluded that they are so generally present in living tissues of higher plants that they establish the plant as an entity of interconnecting protoplasts. In such a system, movement of a virus may proceed through parenchyma tissue at a more or less uniform rate in all directions in which cells are available for invasion. This concept of movement is in harmony with the type of radial spread of viruses observed in inoculated leaves of many different kinds of plants.

Movement in xylem.—Movement of most viruses in xylem has received very little attention and has been considered incidental to the general invasion of the plant and, where it occurs, confined to living cells.

The existence of two viruses, Pierce's disease virus of grape and phony virus of peach, which apparently are restricted largely or completely to the xylem tissue, indicates that this part of the vascular system of plants may have greater significance as a region of virus multiplication and movement than has been indicated from studies of viruses of other types.

Very little is known regarding the parts of the xylem invaded by Pierce's disease virus, beyond the fact that the natural vectors must feed in the xylem in order to produce infection, and that the virus may be introduced into the tracheae in leafhopper feeding. By first allowing viruliferous leafhoppers to feed on a restricted stem area and then making cuttings of stems above the point of feeding, Houston, Esau & Hewitt (32) found that the virus moved upward in alfalfa stems a distance of approximately 10 in. per hr. They concluded that the movement of the virus upward in the stem may be associated with the movement of water in the xylem.

The apparent restriction of peach phony disease virus (33, 34) to the

woody cylinder, the greater concentrations of virus in the root system, and the erratic and uncertain occurrence of virus in the tops of infected plants indicate tissue restrictions to an unusual degree, and it is difficult to account for the invasion of host plants by this virus on the basis of known facts.

It seems improbable that movement through parenchyma cells at the usual rates of travel through such cells could account for the distributions observed, even in incubation periods of 18 months, or more, required for the production of symptoms. It also seems unlikely that the virus moves in the tracheae since travel in such cells should result in more complete invasion of the tops than has been observed.

Hutchins & Rue (91) found that the virus is inactivated in young peach trees by immersing them for 40 min. in water maintained at 48°C. Air temperatures in this range occur in the areas where phony disease is prevalent but it may be doubted whether stems of trees often reach this temperature in the orchard. Prolonged exposure at lower temperatures might tend to reduce the virus content in the tops during the summer season. If, however, the virus moves in the xylem, reinvasion by virus from the root system should take place rapidly and virus should be present in the top during most of the year, which does not appear to be true.

Other possibilities for travel of phony disease virus have been opened up by the discovery by Turner (92) that the virus is spread under orchard conditions by at least four species of leafhoppers, which feed on twigs. Hutchins, Cochran & Turner (93) state that such information as is available on the feeding habits of the subfamily to which these vectors belong is not at variance with the data suggesting a localization of the virus in the woody cylinder.

If the vectors of the phony disease virus feed on the woody cylinder, they probably introduce virus into all of the types of cells of this tissue. Virus introduced into the tracheae would move upward in the transpiration stream, but downward movement would be difficult. Virus introduced into the xylem parenchyma presumably would increase and spread at the slow rates possible in parenchyma tissue. It would seem that in either case the virus would have great difficulty in reaching the root system which is the part of the plant most extensively invaded under natural conditions. However, if it were assumed that the virus may escape occasionally into the phloem and that it is unable to increase there, the observed distributions of the virus in diseased trees could be explained more readily. Virus that escaped into the phloem would move into the root system very quickly to be retained in the phloem of the root until it passed into adjacent wood parenchyma or became inactivated. In this way the root system might be invaded extensively and rapidly, whereas virus in the top would be restricted to regions of the woody cylinder around points of original introduction, and possibly to occasional regions of woody cylinder where virus might escape into new areas of the stem during downward movement to the root system.

Movement in phloem tissue.—After viruses enter phloem from adjacent

parenchyma, or are introduced into the phloem directly through the agency of an insect vector, most evidence indicates that the virus moves rapidly away from the point of entrance toward growing points or other regions of food utilization.

The accuracy with which virus movements in the phloem may be measured varies with different viruses. Accurate measurement of rates of movement of mosaic viruses is difficult because it usually is necessary to introduce these viruses into superficial cells, and entrance into the phloem is effected only after a period of relatively slow and variable movement through parenchyma. However, tobacco mosaic virus (94) appears to be able to move 36 in. in 72 to 77 hr., or at a rate of about ½ in. per hr., in stems of *N. glauca;* Kunkel (49) estimated that it may move as fast as 7 in. per hr. in tomato stems. Capoor (50) found that the viruses of tomato aucuba mosaic, tobacco mosaic, and potato virus X move at about the same rates in tomato stems. He estimated that they may travel more than 80 cm. in 12 hr.

The movement of some of the leafhopper-transmitted viruses through the phloem can be measured with considerable accuracy, since the vectors are able to introduce the viruses directly into vascular tissue. The viruses that have been found to move most rapidly are those causing streak disease of maize and curly top of sugar beet. Storey (95) found that the streak virus actually moved a distance of 3 cm. in 2 hr. in a maize leaf and, as cited earlier, the virus of curly top (38) was found to move 6 in. in 6 min. in a beet leaf.

The specific cells of the phloem through which rapid virus movement occurs have not been determined with certainty. There is evidence, however, that viruses may not be closely confined to protoplasm in this tissue. Sugar beet curly top virus (38) is present in high concentrations in exudate from cut surfaces of diseased beet roots and in droplets often found on the petioles and leaf blades of infected plants. Exudate from severed vascular bundles appears to be derived directly from the phloem. Esau (96) states that the exudate that often appears on the surface of beet leaves affected with curly top may be traced back to the phloem from which it escaped through intercellular spaces in the mesophyll. She concluded that the natural exudate found on the surface of diseased leaves probably is the material translocated in the phloem. This evidence points to the conclusion that viruses occur in the liquid content of the phloem and that rapid transport takes place in this medium. From this evidence, and from the lack of evidence for rapid movement in parenchyma cells of any type, it seems probable that sieve tubes may be the avenues of rapid virus transport in the phloem.

The factors involved in the movement of viruses through the food-transporting system of the plant evidently provide for very rapid virus transport. There is strong evidence in support of the concept that movement tends to be unidirectional and correlated with the translocation of carbohydrates, but this concept has not yet won full acceptance.

Bennett (51) reported that virus of raspberry leaf curl usually is found

only in the inoculated cane of the raspberry plant during the first season of infection. It was induced to enter other canes, however, by defoliating them. Under normal conditions the virus entered the noninoculated canes shortly after growth started the following season. Thus, virus invasion appeared to be correlated with normal carbohydrate transport.

Holmes (97) studied the movement of tobacco mosaic virus away from primary lesions in tobacco leaves, using the starch-retention technique, and found a definite correlation between virus movement and food translocation.

Evidence indicates that a high degree of control of the movement of the curly top virus (83) can be achieved in multiple-crown beet plants by regulating the relative amounts of photosynthetic activity in the respective shoots. In tests with such plants, a single shoot of a plant with three shoots, all of about equal size, was inoculated with curly top virus by means of beet leafhoppers. At the time of inoculation one of the remaining shoots was defoliated. The remaining noninoculated shoot served as a check on the effect of defoliation on the movement of virus from the inoculated shoot into other parts of the plant. Results of a number of tests with three-crowned beets showed that symptoms appeared on the inoculated shoot in average periods of 9.4 to 13.5 days, on the defoliated shoot in average periods of 10.6 to 16.6 days, and on the nondefoliated, noninoculated shoot in average periods of 55 to 183 days. Darkness produced essentially the same results as defoliation. Bawden (98) suggested that in these tests the virus may have entered the check shoots as soon as it entered the defoliated shoot but required defoliation or darkness to produce expression of symptoms. This, however, seems somewhat improbable in view of the fact that symptoms appeared after the usual incubation period in all of the leafhopper-inoculated shoots which were maintained under the same environmental conditions.

Studies of the movement of tobacco mosaic virus (94) and beet curly top virus (83) in tobacco have also indicated a correlation between virus movement and food translocation. In vegetative plants of Turkish tobacco having a main stem in a horizontal position and a basal sucker in a vertical position, basipetal movement of tobacco mosaic virus in the main stem was rapid and acropetal movement was slow. In similar plants maturing seeds on the main stem, acropetal movement was rapid.

In plants of *N. glauca* having top and basal grafts of Turkish tobacco separated by 3 ft. of stem, virus moved from the top graft to the basal graft and produced symptoms in six to nine days. In seven of ten plants the virus failed to move from the basal to the top graft in periods ranging from 224 to 252 days. Movement from basal to top shoot was rapid, however, following defoliation of the top shoot. Essentially the same results were obtained in the studies of the movement of curly top virus in this type of plant.

Samuel (48), Kunkel (49), and Capoor (50) studied the movement of viruses in tomato plants following sap inoculation of a single leaf near the middle of the plant. The viruses required periods of between 42 hr. and 5 days to move out of the inoculated leaf into the stem. Movement was rapid

after the virus entered the stem. Movement was first only downward in some plants, only upward in others, and in both directions in still other plants. Kunkel concluded that movement in both directions in the stem indicated simultaneous movement in two directions but not necessarily in the same sieve tubes. However, it seems possible that movement may have been first in one direction and then in the other, since the virus was estimated to be traveling as fast as 7 in. per hr., and there was no way of determining the exact time of entrance of virus into the stem. Samuel found that fruits and immature leaves were invaded first while movement into mature leaves was greatly delayed. Capoor found that when three viruses were introduced simultaneously into the same leaf, they moved in the same direction and at approximately the same rate.

Movement of viruses out of root systems of inoculated plants appears to be very slow. Tobacco mosaic virus (94, 99) remained in the root system or moved into the tops only after long periods. Removal of tops hastened upward movement. Roberts (100) found that the viruses of bushy stunt, tobacco mosaic, and potato X disease were sometimes localized around the points of initial entrance into the roots, and sometimes they produced systemic infection of roots and tops. In some instances, systemic infection was more frequent when the upper tap root or superficial roots were inoculated than when fibrous roots were inoculated. In general, the evidence from root inoculations of plants with viruses indicates that viruses do not move readily out of root systems, which appears to be in harmony with the concept that virus movement is correlated with food transport.

Apparently, viruses do not readily move out of etiolated parts of plants under conditions of darkness. Curly top virus (83) was retained in etiolated beet leaves in the dark for a period of 21 days but moved out of green leaves in 4 hr. After the etiolated leaves were placed in the light, virus moved out in 24 to 72 hr. Symptoms appeared on young leaves of some of these plants in 4 to 9.5 days after they were returned to the light. These incubation periods are shorter than those found in leafhopper-inoculated plants of the same age which suggests that the virus may have increased in the etiolated leaves during the period in darkness and later moved to the growing point in quantities sufficient to produce symptoms in periods appreciably shorter than those normally required.

In tests with tobacco plants in which a main shoot was maintained in a dark box and a basal shoot in the light, tobacco mosaic virus (94) did not move out of the etiolated shoot in the dark in a period of 40 days, whereas in comparable check plants in the light, virus moved from the inoculated top leaves and produced symptoms in the basal sucker in an average time of 5.2 days.

Although there is evidence that viruses are retained by etiolated leaves in the dark, all available evidence (83, 101, 102) indicates that when viruses are introduced into mature green leaves in the dark, they move out of these leaves as readily as they move out of comparable green leaves in the light.

Moreover, viruses show no greater tendency to move into darkened leaves than into leaves of the same age in the light. These results have been interpreted as opposing the concept of a correlation between virus movement and food transport. However, it may be pointed out that usually mature green leaves deteriorate rapidly in the dark and leaves of many plants die in a short time. It is possible, therefore, that translocation in the phloem of such leaves may be largely outward due to the transport of products of degeneration.

Roberts (102) studied movement of three sap-transmissible viruses in tests designed to eliminate reversals of flow of photosynthates in the phloem and the uncertainty of movement of sugars into darkened green leaves. Plants were kept in the dark three days to deplete them of starch before inoculation. Leaflets of bean and leaves of tobacco were inoculated and the inoculated parts of some of the plants were kept in darkness and others were exposed to light. Virus seemed to move out of the darkened leaflets and leaves as rapidly as from the corresponding parts in the light, but apparently in lesser quantities. It moved out of darkened leaves in which starch was formed along the veins during the period in darkness.

In further tests, tobacco plants were kept in darkness three days; the stems were then scalded at the base to prevent movement of food materials into the roots. A basal leaf on each plant was inoculated with one virus and a top leaf was inoculated with another virus and the plants were kept under constant illumination. Tests of virus content showed that, in most instances, each virus invaded both the tops and basal portions of the plant in periods of 10 to 19 days.

The results of these tests clearly do not indicate a correlation between virus movement and food transport. If it could be demonstrated conclusively that food translocation in the test plants was constantly in a single direction throughout the entire period of test, the results would provide strong evidence against a correlation between virus movement and food transport. However, even with the precautions taken, it does not seem certain that flow of food materials was necessarily constantly in a single direction in all parts of the plant throughout the period of test. Since viruses increase rapidly and may move several inches in an hour, reversals of food flow for short periods would be sufficient to provide for the distributions observed if movement is correlated with food transport. Even short repeated reversals might carry the virus for considerable distances since the virus would be expected always to move from the point of greatest advance in each successive reversal. It would be of interest to repeat these tests with viruses that can be introduced directly into the phloem and measure movement of virus during the first few hours following introduction of virus instead of virus distribution after several days.

The factors involved in the transport of carbohydrates, as well as other materials in the phloem, is still a subject of debate among plant physiologists. Crafts (4) published a complete and comprehensive review of the literature dealing with the movement of assimilates, viruses, growth regulators, and chemical indicators in plants in which he pointed out a similarity in direc-

tional movement and in rates of movement of these different substances. This evidence suggests a common mechanism in the transport of a wide range of materials in the phloem.

The information regarding movement of viruses in plants is still too incomplete and contradictory to justify definite conclusions regarding the mechanism involved in the transport of viruses long distances in short time intervals. However, it appears certain that the rapid movement that occurs in systemic invasions of plants by viruses takes place in the phloem. Although it has not been shown that virus movement in the phloem is always unidirectional, evidence showing that some viruses may move long distances in one direction in plant stems in periods of a few hours or days and fail to move an equal distance in the opposite direction in similar stems in periods of months, seems to demonstrate conclusively that movement is unidirectional under some experimental conditions. Further critical tests are required to determine whether, under other conditions, simultaneous movement in two directions may occur. Such information would have an important bearing on determination of the type of mechanism responsible for the movement of viruses in the phloem and probably also on the mechanism responsible for the movement of many other substances that are transported in the phloem.

If viruses are able to move simultaneously in two directions through the phloem, the protoplasmic-streaming hypothesis favored by Curtis (103) and advanced by Roberts (102) to explain virus movement in tests with bean and tobacco, would seem adequate to explain directional movements. However, movement of viruses in parenchyma, presumably by this mechanism, are far below those that occur in the phloem. Moreover, streaming of protoplasm in the sieve tubes has not been observed. If it occurs, streaming would need to be at an extremely rapid rate to provide for movements of tobacco mosaic virus of $\frac{1}{2}$ to 7 in. per hr., and movements of curly top virus up to 1 in. per min. that have been shown to take place in newly infected plants.

If virus movement is unidirectional, as much of the evidence indicates, protoplasmic streaming could hardly be involved as a major factor in movement, since simultaneous two-directional movement is inherent in the protoplasmic-streaming theory. The theory of transport of materials in the phloem that seems to best conform with the observed facts of virus movement is that proposed by Münch (104) and by Crafts (105); it postulates a mass flow of liquid content of the phloem from the regions of carbohydrate supply toward regions of food utilization or storage. The massflow theory would appear to provide for rates of movement of viruses more nearly approaching those that have been observed. It would provide also for the rapid movements of viruses in a single direction such as have been observed in a number of instances with several different viruses.

Biology of Plant Viruses in Relation to Grouping

Several attempts have been made in the past to use biological relationships in schemes of classification. These attempts, in general, have been

based on the relationships of viruses to both their host plants and their insect vectors with major emphasis varying, depending on the viewpoint of the one making the classification. To what extent such relationships may be found useful in the delimitation and description of virus groups at different levels will depend on the type of classification atttempted. On the basis of their relationships to both their host plants and their vectors, however, viruses appear to fall rather logically into two reasonably well-defined groups that may merit recognition in classifications in which symptoms and vector relationships are given positions of primary importance. These groups may be characterized as follows:

Group 1. This group includes viruses that induce symptoms characterized by primary disturbances in the vascular system. Symptoms consist of such abnormalities as leaf curling, general yellowing, stunting, or rosetting, but no typical mottling or local necrosis in the parenchyma. Some produce distinct necrosis of the phloem and appear to be closely limited to or more concentrated in this type of tissue. The viruses usually are not transmissible by juice inoculation or are transmitted in this manner only with difficulty. Vectors are leafhoppers, aphids, or, more rarely, other types of insects. Vectors have the following characteristics in common: failure to acquire maximum charges of virus in very short feeding periods on diseased plants, continued acquisition of virus over more prolonged feeding periods, failure to produce infection in very short feeding periods, and production of increased infection with increased feeding time. The viruses are retained by their vectors for prolonged periods, sometimes for the life of the insect, and some increase in their insect vectors. There is evidence that these viruses are ingested by insects, pass through the wall of the alimentary tract, and enter the blood and pass into the salivary glands from which they are reintroduced into the plant along with salivary secretions.

Group 2. Viruses of this group induce symptoms characterized by production of primary disturbances in the parenchyma. Symptoms consist of typical mottling, ringspots, local yellow or necrotic areas, or similar disturbances in parenchyma. They usually do not produce obvious symptoms in the phloem. Many are readily transmissible by juice inoculation. Vectors are aphids, or, more rarely, other types of insects. Usually these viruses are acquired in feeding periods of a few seconds; prolonged feeding does not increase the transmissive efficiency of the vector, and the viruses are lost by the insect in a relatively short time. There is no evidence that the relationship of the virus to the vector is one in which the virus requires passage through the insect. There is considerable evidence indicating that transmission is effected by mechanical transfer of the virus from one plant to another on the mouthparts of the insect.

Often it is not possible to draw sharp lines of separation between groups in classifications of biological entities. This is true also with these two groups of viruses, and there are certain viruses that have some characteristics of each group. For instance, euphorbia mosaic virus (106) produces chlorotic

areas in parenchyma and is juice-transmissible, but it is persistent in its insect vector, *Bemesia tabaci*. Similar relationships are found with tomato spotted wilt virus but, in this case, often there is considerable yellowing of infected plants. However, the number of viruses that have characteristics of both groups seems to be limited, and it would appear that the great majority of viruses may be assigned readily to one or the other of these two groups on the basis of their relationships to their host plants and insect vectors.

It seems possible that further evidence of differences between these two groups of viruses may be found with more extensive investigation. As has been indicated already, the viruses of the first group may have a close relationship with the phloem, whereas those of the second group may be more closely associated with various types of parenchyma. Obviously, these two types of tissues present very different media for virus increase. One marked difference is in hydrogen-ion concentration. Fife & Frampton (107) showed that the phloem region of the sugar beet petiole has a pH value well above pH 7.0, whereas the surrounding parenchyma is acid in reaction. This difference in reaction between the two types of tissues seems to be general in plants.

There is evidence also that the blood and salivary secretions of insects are alkaline. It seems probable, therefore, that viruses that have become adapted to increase in phloem tissues of plants may have found the alkaline reactions of body fluids of insects favorable for prolonged active existence and favorable for increase in some instances, whereas those viruses that increase in an acid medium of the type provided in parenchyma tissues of the plant would find the body fluids of insects less favorable for continued activity. Thus, the similarity in reaction of phloem tissues and body fluids may be involved in the ability of some viruses and in the inability of other viruses to persist in their insect vectors.

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IRON CHLOROSIS[1,2]

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Chlorosis is a general term which denotes a lack of chlorophyll, a condition related to a large number of abnormalities in plants. The more specific term, iron chlorosis, refers to chlorosis which can be alleviated by providing the plant with suitable iron compounds; the plant with iron chlorosis is therefore said to be iron deficient. Iron deficient plants may be found on both acid and alkaline calcareous soils in various parts of the world. This disorder is particularly prevalent on calcareous soils where it is difficult to keep iron in forms which are available for all plant growth. Bicarbonate ion concentration, macro- and microelement imbalance, and the ionic iron species in the growth medium all appear to have some effect upon the capacity of the plant to utilize the iron in a soil.

Plant species grown under a specified environment differ in their susceptibility to chlorosis. They also differ in the concentration of iron required in the nutrient medium for their growth processes and production of chlorophyll. The most urgent problems confronting the investigator appear to be: (*a*) an understanding of the biochemistry in chlorosis-susceptible and nonsusceptible plants, (*b*) a resolution of the complex interactions of causative factors, and (*c*) development of more effective means of correcting or controlling chlorosis. It is the purpose of this paper to review some of the published data pertaining to iron chlorosis which may contribute to resolving these problems. For comparative purposes, a limited amount of data are included on iron metabolism in animals.

[1] The survey of the literature pertaining to this review was concluded in October, 1955.

[2] The following abbreviations will be used: APCA, aminopolycarboxylic acid; CDTA, cyclohexane-trans-1,2-diaminotetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; HEEDTA, hydroxyethylethylenediamine triacetic acid.

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Functions of Iron in Plants and Animals

Depending on the pH of the medium, inorganic iron salts possess the properties of an oxidase and of an electron transporter, but their capacity to function as a catalase or peroxidase is very slight (26). When iron is incorporated into the porphyrin ring, the catalase and peroxidase activities are enhanced. When the iron porphins become attached to specific proteins, not only do these particular protein compounds acquire marked degrees of specialization and enormous enhancement of activity but the hemes are also stabilized and protected. Depending on the protein to which it is attached the heme may function as a transporter of molecular oxygen, transporter of electrons, activator of oxygen, activator of H_2O_2 or decomposer of H_2O_2 (80). Porphyrins by themselves show no catalytic activities (26). It is only when the porphins form metal coordination compounds that they appear to develop properties of great physiological significance. Although a large number of metals can be incorporated into the porphyrin ring, only two are of importance biologically, namely, the iron porphins or heme compounds and the magnesium phorbins or chlorophylls. Copper and zinc porphins have been reported in traces and under special conditions.

The importance of iron enzymes in respiration has been thoroughly established. Most plant and animal cells contain the cytochromes (23) which consist of a number of iron porphin enzymes distinguishable by their absorption bands. Some of the cytochromes are oxidases and function as activators of atmospheric oxygen, converting it to a powerful electron acceptor. Certain of the other cytochromes are electron transporters and act as intermediates shuttling electrons from the substrate to the oxygen-activating catalysts (26).

Webster (98, 99) extracted an enzyme from 54 different plant species representing 23 families of dicotyledonous plants which oxidized cytochrome *c* and which was strongly inhibited by carbon monoxide. James (44) has reported the disappearance of the cytochromes in barley roots after ten days growth and the appearance of a respiratory enzyme which is inhibited by carbon monoxide, but is not reactivated by light. He concluded that ascorbic acid oxidase may be serving as the terminal oxidase in these roots. Waygood (97) suggests ascorbic acid oxidase as the terminal oxidase in wheat. To what extent terminal oxidases, other than the cytochromes, may serve in plant respiration needs clarification.

Iron Supply

The formation of chlorophyll and hemoglobin, two of the most important pigments known in plants and higher animals, is dependent upon an adequate supply of iron. In their natural environment, the inherent supply of iron is usually adequate for both plants and animals. For plants, actual deficiency of iron in the nutrient medium is probably found only in sand or water cultures (79). The capacity of the plant or animal to utilize its iron supply is our chief concern in iron chlorosis of plants or hemoglobin formation in animals. Before discussing the plant aspects of this iron supply problem it

might be well to comment briefly on some of the factors which influence iron in animals.

The total amount of iron in the human body has been generally estimated at between four and five gm., of which 65 to 75 per cent is combined with porphyrins and the rest is in inorganic form bound to proteins (20). Absorption of iron is dependent upon a regulatory mechanism which controls the passage of iron across the mucosal cells of the intestinal tract in accordance with the needs of the body.

Granick (24) suggests that ferrous iron in the mucosal cell is oxidized to the ferric condition and becomes attached to apoferritin to form ferritin. The ferric iron (*a*) is reduced to the ferrous condition, (*b*) is detached from ferritin, and then (*c*) enters the blood stream where it is autoxidized and attached to siderophillin for transport. The iron-binding property of siderophillin is specific for this particular protein. Two regulatory mechanisms exist: (*a*) absorption of iron into the cell is controlled by the content of ferritin, and (*b*) release of iron into the blood stream is dependent upon the oxygen level of the blood. When the latter is low, conditions favor the formation of ferrous iron and its transfer to the blood stream. A greater reducing tendency or lower "redox" level would cause more iron to pass into the blood stream. Such a lower redox level might arise either by an increased effectiveness of the reducing enzymes or by a decrease in the effectiveness of the oxidizing enzymes (25).

Hemochromatosis is a heritable disease of metabolism characterized by an extensive deposition of iron throughout the body. It appears to be caused by a decrease or absence of regulation of iron absorption by the mucosa.

Iron absorption by the body is decreased by phosphates in the diet and is also related to the ratio of Ca to P. Where the ratio is low, phosphate combines with the iron rendering it insoluble and relatively unavailable for absorption (20). Accordingly, the amount of iron deposited in the liver of rats is inversely related to the phosphorus content of the diet.

Copper is not a constituent of the hemoglobin molecule, but Elvehjem & Sherman (18) showed that copper is indispensable for the incorporation of absorbed iron into the blood hemoglobin. It does not influence iron assimulation (56). Copper has also been shown to be essential for the formation of cytochrome oxidase and catalase and for maintenance of their activity in certain rat tissues (20). Shultze & Kuiken (74) showed that in the liver, kidney, and blood of copper-deficient rats the catalase activity was very low. Feeding of copper to the deficient rats induced a rapid return to normal catalase activity. The above findings indicate that copper is involved in the iron metabolism in animals.

Since iron is not deficient in most soils, variations in the capacity of plants to absorb and accumulate this element must be due to factors other than the level of iron in the soil. Thus, there appears to be a regulatory mechanism for the absorption and translocation of iron in plants similar to the mechanism proposed in animals.

Most iron compounds occurring naturally in soils are quite insoluble.

Ferrous ions are unstable in aerated soils with a pH value of 6.0 or higher. Alkalinity and aeration favor oxidation of iron and water-logging favors reduction (50). The extent of oxidation or reduction is also influenced by other factors, such as activity of micro-organisms, level of soil organic matter, and the presence of other cations or anions. Kliman (47) stated that bivalent iron is the form of iron best absorbed by plants.

It must be recognized that some plants will grow well where others will not grow without producing iron chlorosis. This has been recognized as differences among plant species, but very little work has been done to explain these differences. It would appear to be of great importance to determine why different plant species grown under a specific environment differ in their susceptibility to chlorosis.

Brown (9) grew a number of plant species on calcareous and organic soils to test their susceptibility to chlorosis (iron deficiency) and copper deficiency. The plants responded variously (see Table I). There were: (*a*) those which

TABLE I

PLANTS GROWN ON A CALCAREOUS AND ORGANIC SOIL, AND THE OCCURRENCE OF LIME-INDUCED CHLOROSIS OR COPPER-DEFICIENCY SYMPTOMS, RESPECTIVELY

Plants	Calcareous Soil (lime-induced chlorosis)	Organic Soil (copper-deficient systems)
1. Thatcher wheat	0	+
2. Hanchen barley	0	+
3. Long green okra	0	+
4. Midland milo	0	+
5. Cocklebur	0	+
6. Red kidney beans	+	0
7. White lupine	+	0
8. Blue lupine	+	0
9. Yellow lupine	+	0
10. PI-54619-5-1 soybeans	+	0
11. Caloro rice (upland)	+	0
12. Connecticut broadleaf tobacco	+**	0**
13. Marglobe tomato	+**	0**
14. Minnesota hybrid #800 corn	+**	0**
15. Ranger alfalfa	+**	0**
16. Hawkeye soybean	0	0
17. Ottawa select spring rye	0	0
18. Utah 10B celery	0	0
19. Summer pascal celery.	0	0

+ = visual deficiency symptoms other than retarded growth.
0 = no visual deficiency symptoms.
** = partial deficiency symptoms.

did not develop copper-deficiency symptoms when grown on an organic soil low in available copper but did develop iron-deficiency chlorosis on the calcareous soil; (*b*) those which developed copper-deficiency symptoms when grown on the organic soil but did not develop iron-deficiency chlorosis on the calcareous soil; (*c*) those which showed retarded growth and slight deficiency symptoms in some cases when grown on both the calcareous and organic soils; and (*d*) those which showed no chlorosis or deficiency symptoms when grown on either soil. Comparative activities of ascorbic acid oxidase and catalase were measured in all of these plants (9, 10).

Generally speaking, the comparative determination of ascorbic acid oxidase activity showed that plants (Table I) grown on the calcareous soil had an ascorbic acid oxidase activity comparable to that of plants grown on the organic soil containing 400 p.p.m. of added $CuSO_4 \cdot 5H_2O$. Also, the activity was 30 to 40 times greater in most of these plants than in plants grown on the organic soil to which no copper had been added. These results reflected the degree to which the supply of active copper was limited in these plants. They did not all develop externally visible deficiency symptoms even though the copper supply was limited; internally, differences were very apparent.

Catalase activity was much greater in copper-deficient than copper-sufficient corn, tobacco, and tomatoes. There was a marked accumulation of iron in the nodes of deficient corn plants. It appears that copper may be a part of a regulatory mechanism for iron in corn. Triphenyltetrazolium chloride was much more readily reduced in corn plants containing sufficient copper than in those deficient in the element (8). This indicates a possible change in the effectiveness of the reducing or oxidizing enzymes. Such a condition could be expected to affect the valency of iron and its absorption and transport in the plant. More work needs to be done to clarify these observations.

In some iron absorption studies involving the plants listed in Table I the PI-54619-5-1 soybean (*Glycine max*), Thatcher spring wheat (*Triticum sativum*), Minnesota hybrid No. 800 corn (*Zea mays*), and Hawkeye soybean (*Glycine max*) were selected to represent each group of plants, respectively. Only the PI-54619-5-1 soybean failed to absorb sufficient iron from a calcareous Millville soil to maintain growth. Autoradiographs confirmed these observations (11). From the above observations it would appear that the availability of any supply of iron in the growth medium to plants will be dependent upon both the plant species and the growth medium.

Iron Requirement

Iron occurs in rather large amounts in nearly all soils, but it is present only in relatively small amounts in plant and animal tissues. This presents some difficulties to the analyst, since contamination may introduce quantities of iron in excess of those in the tissues. Soil, dust, and spray residues may be quite difficult to remove from surfaces of plant material. This is

particularly true for pubescent plants. Inadequate washing of the sample has been shown by Jacobson (42), Wallihan (94), and Smith *et al.* (77) to result in erroneously high values for the iron content of plants.

Glenister (22) found that the lower older sunflower leaves contained about 10 times as much iron as the younger upper leaves. He concluded that iron is not transferred from the older to the younger parts, and that a continuous supply of iron is necessary if the younger leaves are to appear normal in color and have a normal respiration rate. Iron deficiency resulted in both a chlorosis and a reduced respiration localized in the younger leaves. Other studies (11), using radioiron have indicated that a continuous source of available iron is necessary to prevent chlorosis in the younger leaves of soybean plants.

If the age of the leaves sampled and the removal of surface contamination are thoroughly considered in determining iron in plant material, it might be possible to come to better agreement in the literature on the iron content of chlorotic and nonchlorotic plant tissues. Some workers (1, 41, 92, 104) have failed to find consistent differences in iron concentrations in chlorotic and healthy leaves. Wallihan (94) concluded that iron chlorosis of citrus is caused simply by a deficiency of iron in the leaves, and that chemical analysis can be a useful tool in studies of iron nutrition of citrus provided contamination of leaves is avoided. It is the opinion of the author that this may not be true in some plant species or under certain conditions of growth, and emphasis should not be placed on this point until more is known about the mechanism by which iron absorption and translocation are regulated.

Thorne *et al.* (87) report that green leaves washed consecutively in a detergent solution, dilute HCl, and distilled water, consistently contained more iron than similarly washed chlorotic leaves from the same area. However, the iron in the leaves was found to be quite variable from orchard to orchard, and it was not possible to determine any critical value which would consistently differentiate green from chlorotic leaves. Kuykendall (49) made a survey of Florida citrus in which he collected leaf samples from 123 groves in 21 counties. Leaf iron was divided into three categories: (*a*) below 60 p.p.m. iron was considered low (leaves possibly chlorotic); (*b*) between 60–90 p.p.m. iron was regarded as medium; and (*c*) any values above 90 p.p.m. iron were classified as high. The mean leaf iron content for all varieties in all locations was 66.6 p.p.m. and in 41 per cent of the groves the iron content was below 60 p.p.m. Leonard & Stewart (52) found that mildly chlorotic leaves contained about 55 p.p.m. iron. The critical concentration of iron in citrus leaves was determined by Wallihan (94) to be about 80 p.p.m.

Noack & Liebich (63) report that 82 per cent of the total iron in spinach leaves is associated with the chloroplasts. They separated this iron into various fractions and concluded that five-sixths of the firmly bound iron is absorbed on phosphorus-containing proteins. Liebich (53) showed that the cell nucleus contained only traces of iron, and the cell wall no iron. Three

fractions of the chloroplastic iron in spinach leaves were differentiated: (*a*) 8 per cent H_2O-soluble iron; (*b*) 32 per cent that can be removed by 0.01 *N* HCl or 0.001 *N* KOH; and (*c*) 60 per cent residual iron. Chloroplasts represent about one-third of the dry weight in sugar beet leaves and contain 61 per cent of the total iron (103).

The amount of iron absorbed by isolated chloroplasts from wheat is reported by Hagen *et al.* (27) to be governed by at least three factors: (*a*) the mass of chloroplasts; (*b*) the concentration of iron in the system; and (*c*) the iron-binding capacity of the suspension medium. A change in any of these factors may influence the absorption of iron by the chloroplasts. Mitochondria introduced into a chloroplast suspension containing acetate and Fe 55–59 diminished iron absorption by the chloroplasts of wheat. This indicates a possible competition for iron between different plant particulates, which might affect the availability of iron in immature leaves. This effect becomes less important as the plant matures, and the mass of the chloroplasts is relatively large in comparison to that of the other particulates in the cell. Jacobson (42) concluded from studies on tobacco leaves that "active" iron was localized in the chloroplasts and that nonchloroplast iron could accumulate only when iron was no longer a limiting factor in chlorophyll formation. He agreed to the term "active" iron proposed by Oserkowsky (68) as that portion soluble in 1 *N* hydrochloric acid. Bennett (3) found that the active iron content of pear leaves built up rapidly early in their growth and then diminished, whereas residual iron was low at first and then accumulated as the growth progressed. He concluded that "active" iron can be transformed into residual iron, but that iron once transformed into the residual state cannot be utilized for chlorophyll production. This might be a failure of iron to be translocated rather than the capacity of iron to function in chlorophyll formation. Brown & Holmes (11) observed that iron was mobile in green leaves of soybeans with one source of iron but was immobile to chlorotic leaves which developed on the same plant when the plant's source of iron was changed.

Waring & Werkman (95) observed that in iron-deficient bacterial cells of *Aerobacter indologenes* certain iron-containing enzymes may be depleted or entirely absent without affecting the activity of other enzymes present. There appeared to be a selective distribution of the iron in the deficient cells. The iron requirement of the cytochrome system appeared to be met first. Earlier, Waring & Werkman (96) had determined the iron requirement for the growth of six bacterial species and found it to vary with the type of cytochrome system contained in the organism. The iron requirement of one of the organisms appeared to be about three to four times greater than that of the other organisms studied. This organism contained the four band cytochrome system, had higher catalase and peroxidase activities, and showed stronger hematin tests than other bacteria. An incomplete or primitive type of cytochrome system appeared to exist in some of the other organisms.

Holmes & Brown (34) found the iron requirement of two soybean varieties to differ when grown in solution cultures at different concentrations of iron. The chlorosis-susceptible variety required a higher iron concentration to produce green plants than the nonsusceptible variety. Weiss (102) showed that the difference in efficiency of iron utilization between the chlorosis-susceptible and nonsusceptible soybeans was conditioned by a recessive gene. The iron requirement of a plant appears to be a factor in its susceptibility to iron chlorosis.

Causative Factors Related to Iron Chlorosis

Iron chlorosis has been observed in plants growing on both acid and alkaline soils. Leonard & Stewart (52) state that three-fourths of Florida's citrus is grown on acid soils and trees showing iron chlorosis are widely distributed on them. These soils are very sandy, are low in exchange capacity, and have a low fertility level. Reuther & Smith (72, 71) and Smith & Specht (75) concluded from greenhouse and field studies that the acid soil chlorosis of citrus in Florida may be caused in many instances by a high concentration of available heavy metals (especially copper) in relation to available iron in the soil.

The most widespread incidence of iron deficiency in plants occurs on alkaline calcareous soils where the abnormality has been termed "lime-induced" chlorosis. To explain the relation of observed facts to the occurrence of this chlorosis Thorne *et al.* (87) discussed the following hypotheses:

1. High pH and excess quantities of lime make soil iron less available to plants. 2. High soil moisture, poor aeration, and cool temperature disturb plant metabolism so that iron is inactivated (13). 3. Phosphates may precipitate iron either in the soil or in plant tissues (4). 4. High manganese in soils or plants may oxidize iron to an inactive state (78). 5. Excessive amounts of potassium in leaves may displace iron from an active enzyme surface and thereby disrupt metabolic processes (46). 6. An unbalanced cation content of plant tissues may lead to disrupted synthetic activities with an abnormal accumulation of certain organic acids and an increase in soluble nitrogen at the expense of insoluble protein (3, 39, 40). 7. Soil and plant conditions associated with excess lime in soil favor the oxidation of iron to the less active ferric state and its fixation in compounds of low biological activity (3, 68, 85). None of these hypotheses unify the observed facts into a rational concept of the disease.[4]

This list of hypotheses might be modified with the understanding that plant species and varieties may differ in their susceptibility to iron chlorosis with each of the following factors: bicarbonate ion; macronutrient elements; micronutrient elements; environmental factors; and iron status in the growth media.

Bicarbonate ion.—Bicarbonate-ion-induced iron chlorosis has received considerable attention since the observations made by Harley & Lindner

[4] The bibliographic reference numbers in this quotation have been changed to correspond to the reference numbers used in the bibliography of the present review.

(28) on the responses of apple and pear trees to irrigation waters which varied in their bicarbonate ion concentrations. Chlorosis in varying degrees was always found associated with soils containing carbonates although no quantitative relationship was found between the percentage of carbonate in any of the horizons and the incidence or degree of chlorosis. The quality of the water used seemed to be an important factor. In areas where chlorosis was most severe the irrigation waters came from wells, springs, or small creeks containing bicarbonates in concentrations of 200 p.p.m. or more. Subsequent irrigation with water low in bicarbonate tended to alleviate the condition.

Heller *et al.* (29) found that sodium bicarbonate produced injuries in tomatoes at lower concentrations and, in general, was more toxic than sodium chloride. Sodium bicarbonate treatments reduced the calcium content in the plants very markedly from one-fourth to one-half that of the controls.

Carnations were observed by Wall & Cross (89) to be virtually insensitive to sodium bicarbonate, whereas chrysanthemums were extremely sensitive. Using solution cultures containing 12 m.e. per liter $NaHCO_3$, Gauch & Wadleigh (19) found that Rhodes grass was insensitive and grew well, whereas this concentration induced severe chlorosis in Dallis grass. The control nutrient solution adjusted to pH 8.0 with NaOH, but nearly free of bicarbonate, did not have a detrimental effect on Dallis grass. They concluded that the bicarbonate ion was specifically toxic over and above the effect of pH values associated with solutions of $NaHCO_3$.

Wadleigh & Brown (88) studied the effect of bicarbonate on dwarf red kidney beans (*Phaseolus vulgaris* L.). They found that 8 m.e. of sodium bicarbonate per liter of nutrient solution produced chlorosis and reduced the growth of the beans by more than one-third. Growth was completely stopped where 32 m.e. of sodium bicarbonate was used. They attribute this effect to the bicarbonate ion which decreases iron availability at the root surface.

Lindsay & Thorne (55) concluded from their aeration-bicarbonate studies that chlorosis is aggravated by high oxygen levels. At low oxygen levels chlorosis was less prevalent. They concluded that chlorosis associated with poor aeration cannot be attributed to low oxygen levels in the growth media. Increasing levels of carbon dioxide at the roots of plants growing in a bicarbonate medium contributed to the chlorotic condition by raising the bicarbonate level. They observed that the bicarbonate ion reduced movement of radioiron into the leaves and stems and accentuated its accumulation in the roots. De Kock (15) essentially repeated the work of Lindsay & Thorne using mustard (*Sinapis alba*) and came to the same conclusions.

Porter & Thorne (69) found that at constant pH, maintained by varying the CO_2 content of the aerating gas, increased $NaHCO_3$ concentrations decreased growth and chlorophyll contents of bean and tomato plants.

Varying the pH with various CO_2 partial pressures at constant $NaHCO_3$ content produced no significant effects upon the plants. Wadleigh & Brown (88) give emphasis to the effect of bicarbonate on the entry and activity of iron, the depressed entry of calcium, and the accentuated entry of potassium into bean plants. Baxter & Belcher (2) suggest that accumulation of HCO_3^- ion around roots unfavorably affects CO_2 excretion and internal pH, and is the main factor in the metabolic disturbance leading to iron deficiency.

Miller (60) observed that $NaHCO_3$ inhibited respiration in excised roots of plants which were known to be susceptible to lime-induced chlorosis, but respiration was inhibited only slightly in plants not susceptible to this chlorosis. Steward & Preston (81) studied the effect of the pH value and components of the bicarbonate buffer system on the entry of bromide into potato discs and found that "the most effective component of the bicarbonate buffer system which suppresses the uptake of bromide is the bicarbonate ion itself." Increasing concentrations of $KHCO_3$ progressively depressed protein synthesis and oxidase activity with a decrease in respiration and metabolism of potato discs.

Studies on soil compaction, poor drainage, addition of green and barnyard manures to soils, and high bicarbonate in irrigation water all indicate that bicarbonate may be a causative factor of chlorosis in calcareous soils. Actually very little data are available which compare the bicarbonate content of soils producing chlorotic and nonchlorotic plants (55); most of the results published involve solution cultures using $NaHCO_3$. This leaves a great deal to be done as far as soils are concerned. It still must be proven whether HCO_3^- ion affects availability of iron in soils directly or indirectly.

Olsen *et al.* (67) used sodium bicarbonate as an extractant for estimating the available phosphorus in soils. The theoretical basis of the method is the premise that the solubility of calcium phosphate, such as dicalcium phosphate or hydroxyapatite, is increased by increasing H^+ activity and decreased by increasing Ca^{++} activity. In a 0.5 *M* solution of $NaHCO_3$, they report the equilibrium calcium concentration as being 857 times lower than in a pure $CaCO_3$—H_2O system in equlibrium with atmospheric CO_2. The main effect of the $NaHCO_3$ in calcareous soils would appear to be one of decreasing the Ca^{++} which, in turn, increases the solubility of phosphorus. The higher phosphate and lower calcium could be responsible for the resultant chlorotic plants. An additional effect of $NaHCO_3$ might be through ionic competition of HCO_3^-, $CO_3^=$ and OH^- for phosphate adsorbed on the surface of soil particles.

It seems reasonable to assume that the bicarbonate ion effect in producing chlorosis might be an indirect as well as a direct effect. Biddulph (5) analyzed the precipitate on root surfaces and found that at pH 7.0 calcium is an important constituent along with phosphorus and iron. More work is needed to show the specific way in which the bicarbonate ion affects iron metabolism.

Macronutrient Elements.—The macronutrients most frequently dis-

cussed as being associated with iron chlorosis are calcium, phosphorus, potassium, and nitrogen. One of the most consistent differences between green and chlorotic leaves is a difference in the calcium/potassium ratio (59, 54, 93). In chlorotic citrus leaves the calcium/potassium ratio is lower and the nitrogen higher than in green leaves (58). Iljin (40) believes that the increase in soluble nitrogen in chlorotic plants results from protein disintegration. Holley & Cain (33) have noted that plants suffering from iron chlorosis tend to accumulate more arginine than nonchlorotic plants.

Drouineau (17) and McGeorge (59) determined "active" calcium in a soil by shaking 10 gm. of soil with 250 ml. of 0.2 *N* ammonium oxalate, filtering, and titrating an aliquot of the filtrate with potassium permanganate. The difference between the original oxalate content and that of the filtrate represented the calcium present in the soil in an active form. This calcium value was higher in soils from badly chlorotic citrus groves than nonchlorotic groves. Thorne *et al.* (87) essentially reached the same conclusions by extracting soils with distilled water.

These results indicate a qualitative difference in soil calcium that is related to chlorosis. The specific role of calcium in causing chlorosis is, however, not yet clear. Calcium may be acting competitively with other elements for sites in specific coordination compounds found in the plant, the extent being determined by characteristics of the plant and soil.

Bolle-Jones (7) found that potato plants grown in a medium at low levels of iron became chlorotic in the presence of calcium carbonate or at high phosphorus levels or when potassium was deficient. He found this chlorosis was cured in each instance by the addition of high levels of potassium which was believed to increase iron utilization in chlorophyll formation. In lime-induced chlorosis, Thorne *et al.* (87) concluded that high potassium in chlorotic leaves and a high potassium/calcium ratio seemed to result from chlorosis rather than be a cause of it.

High levels of phosphorus in the growth medium often have been found to reduce iron absorption and utilization, especially under neutral or alkaline conditions (65). Biddulph & Woodbridge (6) showed that plants grown in media supplying an excess of phosphorus progressively accumulated this element in the leaves, stems, and roots. They believed that iron is so frequently immobilized in this manner that they adopted the term "phosphorus-induced chlorosis" to designate this type of disturbance. De Kock (15) advocates the use of the phosphorus/iron ratio as a means of making an assessment of the iron status of plants.

Brown *et al.* (12) observed that several different plant species which varied in their susceptibility to lime-induced chlorosis also varied in their susceptibility to a copper-phosphorus induced chlorosis. Six levels of copper and five levels of phosphorus were applied to a soil medium. Under the conditions of these experiments increasing additions of phosphorus did not appear to affect the absorption or utilization of iron unless copper was also added to the growth medium. Copper and phosphorus were more effective in produc-

ing chlorosis if applied together, than if either element was applied separately. Some plant species did not develop chlorosis, but grew well and produced maximum yields at the highest copper and phosphorus levels. The concentration of phosphorus was higher in the sap of chlorotic than nonchlorotic soybeans. It did not differ appreciably in the residue of these plants. More phosphorus was absorbed by the plants as the rate applied to the growth medium increased. This resulted in a proportionately greater concentration of phosphorus in the sap than in the plant residue of the chlorotic plants.

Bennett (3) determined the phosphorus and iron content of green and chlorotic leaves of the French prune tree grown in acid and calcareous soils. Variations in phosphorus content were unrelated to chlorosis. Thorne & Wann (86) determined the carbon dioxide soluble phosphate content of high lime soils where chlorosis was severe and in these soils the phosphate varied between 2 and 78 p.p.m. and showed no relation to chlorosis. Heavy phosphate fertilization in orchards where chlorosis was a problem did not increase the severity of the disorder even though leaf phosphate was increased by 30 per cent.

There are sufficient experimental facts available to indicate that phosphorus under some conditions can influence iron metabolism and that plant species and varieties differ in their susceptibility to this effect upon iron. This difference has been observed to exist between two soybean varieties (12) which differ genetically (102) in their susceptibility to lime-induced chlorosis and high phosphorus and copper concentrations in the growth medium.

Micronutrient Elements.—An abnormally high concentration of one, several, or all of the micronutrients, except iron, usually causes an induced iron deficiency chlorosis. The effectiveness of some of the micronutrients to produce chlorosis in oats was found by Hunter & Vergnano (38) to be in the following order: Ni > Cu > Co > Cr > Zn > Mo > Mn. Hewitt (30) showed their effectiveness in sugar beets, tomatoes, potatoes, and kale to be Cd, Cu, Co highly effective, and Ni, CrO_4, Zn, Cr, Mn less effective in the order listed.

Ramirez-Silva (70) found that iron showed no toxic effects on pineapple plants when added as the only micronutrient element to a culture solution up to 5 p.p.m. If manganese and copper were present in the solution, higher concentrations of iron were beneficial. Weinstein & Robbins (101) found that both low levels of iron and high levels of manganese in solution cultures resulted in a low activity of catalase and cytochrome oxidase in the plant. They suggested that both treatments induce similar alterations in the metabolic pattern of these plants. Smith & Specht (76) reported that copper appeared to be about 50 times as toxic as Mn and 12 to 15 times as toxic as Zn in orange and mandarin seedlings grown in vermiculite and solution cultures.

Toxicity attributed to excess of certain micronutrients occurs naturally

in various parts of the world. Pineapples were chlorotic when grown on manganiferous soils (21, 35), while plants grown on soils derived from serpentine rocks developed chlorosis due to excess of nickel (36, 37). Reuther & Smith (71), using sand culture, demonstrated that an excess of copper caused iron chlorosis in citrus. Smith & Specht (75) suggest that the main cause of iron chlorosis in Florida is the accumulation of copper in the soil, applied over several decades in sprays and fertilizers.

De Kock (16) states that

> the toxic effect of metals follows closely the order of stability of their chelate complexes, the metal is evidently combined into some protein chelate in the root. This must moreover be the same protein complex into which iron is bound, so that the metal competes for and displaces iron from active chelating centres. This displacement of iron from the root tissues would then depend on the stability of the metal complex relative to iron and its concentration in solution. Thus when the solubility of iron in nutrient solution was greatly increased by chelation, metal toxicity sharply decreased.

Sufficient experimental data are not available to explain the effect of the heavy metals upon iron metabolism. Interrelationships between elements and degree of iron chlorosis have been discussed with reference to molybdenum and manganese as related to form or supply of nitrogen (62, 31), copper as related to supply of phosphorus (12), and iron and manganese nutrient levels (101) in the growth medium. Plant species and varieties differ so much in their susceptibility to the effect of heavy metals (9, 14, 32, 64) that it is difficult to reach any general conclusions. An understanding of this phase of iron chlorosis is difficult until more is known about how the heavy metals affect iron utilization. For example, of the four plants selected from several groups of plants which differed in their susceptibility to lime-induced chlorosis (9, 12), copper deficiency affected iron metabolism in corn in a different manner than in either PI-54619-5-1 or Hawkeye soybeans or wheat. PI-54619-5-1 soybeans were the only plants which developed an iron deficiency chlorosis when grown at high copper and phosphorus levels. Copper deficiency in wheat and iron chlorosis in PI-54619-5-1 soybeans have both resulted in an accumulation of citric acid in the respective plants.

Environmental factors.—Any effect of light, soil temperature, or soil moisture which affects plant metabolism and the status of the elements in the soil associated with iron chlorosis will either enhance or tend to inhibit chlorosis. Burtch *et al.* (13) report that a high moisture level together with low soil temperature is the condition most conducive to the development of lime-induced chlorosis. Thorne (84) believes that 50 to 100 per cent of all chlorosis in some areas can be eliminated by proper control of soil moisture.

Temperature may affect the uptake of iron by influencing the rate of growth of the plants and the activities of the micro flora in the soil. Jones (45) & Millikan (61) noted that cool temperatures enhanced chlorosis of gardenias and flox.

Iron status in the growth media.—Iron is not deficient in most soils, yet

plant species vary considerably in their ability to utilize the iron present. Olsen & Carlson (66) sampled soils from selected areas of Kansas where sorghum plants exhibited iron chlorosis, from areas free of chlorotic sorghum but having chlorotic trees, and from areas having no iron chlorosis. Soils from areas where the sorghum was chlorotic contained less ammonium acetate-acetic acid (pH 4.8) extractable iron than those producing no chlorosis. Iron-deficiency chlorosis is easily obtained in plants by withholding iron from sand or water cultures. Here also, the kind and concentration of iron required in solution to prevent chlorosis will vary with the plant species or variety (34).

Iron deficiency is perhaps the most difficult of all mineral deficiencies to correct. Some practical methods of control for lime-induced chlorosis have been attempted and practiced for more than 25 years. Iron sprays and injection of trees with iron salts have been somewhat successful in citrus and peach trees. The beneficial effects have lasted for a few weeks to more than a year. Although these methods have been successful in supplying iron to trees, they have not been sufficiently practical for large scale use.

In solution and sand culture work it is difficult to keep iron in solution when applied as an inorganic salt because it precipitates. Jacobson (43) was able to maintain a satisfactory supply of available iron in a nutrient solution by a single addition of ferric potassium ethylenediamine tetraacetate, prepared by mixing a salt of iron with a potassium salt of ethylenediamine tetraacetic acid (EDTA). One mole of EDTA was required to chelate one mole of iron (Fe-EDTA).

Stewart & Leonard (51, 82) were able to correct an iron chlorosis in citrus trees grown on a sandy acid soil by adding Fe-EDTA to the soil. This work has stimulated research on the practical importance of metal chelates for the treatment of micronutrient deficiencies in agricultural crops in all parts of the country. Two good reviews on the use of chelates for growing plants have been written by Stewart & Leonard (83) and Kroll *et al.* (48). The chemistry of metal chelate compounds is covered in more detail by Martell & Calvin (57).

The word chelate comes from the Greek signifying claw and refers to the ring configuration when a metal ion combines with two or more electron donors. There are a large number of chelating agents, but the electron donors which bind to the metals are limited to nitrogen, oxygen, and sulfur (83). After a chelate is formed, the metal ceases to have its ionic chemical properties. Naturally occurring chelates are citric, tartaric, oxalic, and amino acids. Some very selective chelates are chlorophyll, a magnesium chelate, and the cytochromes which are very stable iron complexes.

The chelation reaction proceeds according to the law of mass action (83):

$$\text{Metal ion} + \text{Chelating agent} \rightleftarrows \text{Metal chelate}$$

$$K = \frac{(\text{Metal chelate})}{(\text{Free metal ion}) \times (\text{Chelating agent})}$$

K is the equilibrium constant of the reaction and defines the molar ratio of the metal which occurs in the chelate to the metal in the ionized state. Where K is very large, the metal ion concentration is very small. The K value is commonly referred to as the stability constant of the chelate and will vary depending on the metal, chelating agent, and other factors. Stability constants are usually expressed as the logarithm of the K value; the higher the log K value the more stable is the complex and the lesser the tendency for the complex to yield metal ions.

The properties of chelates vary with pH; for example, above pH 6, the OH^- ion will compete with EDTA for Fe^{+++} until, at about pH 8, EDTA ceases to be a very effective chelate for this metal. The chelate becomes ionized in acid solutions to the extent that practically none of the metal will be complexed. EDTA will remove iron from ferric phosphate; however, since chelation is controlled by the law of mass action, there is in reality competition between phosphate and EDTA for iron. Hence, if the phosphate concentration is high enough with respect to EDTA, some iron phosphate will precipitate in spite of the presence of EDTA.

The chelating agents which have been studied (34, 91) with regard to iron chlorosis are ethylenediaminetetraacetic acid (EDTA), hydroxyethylethylenediaminetriacetic acid (HEEDTA), diethylenetriaminepentaacetic acid (DTPA), cyclohexane-trans-1,2-diaminotetraacetic acid (CDTA), and an aromatic aminopolycarboxylic acid (APCA). Kroll *et al.* (48) report the relative order of decreasing stabilities of these chelates of pH 7 to be as follows:

$$\text{CDTA} > \text{APCA} > \text{DTPA} > \text{HEEDTA} > \text{EDTA}$$

The Fe-CDTA chelate appeared to be the most stable complex that was evaluated in this series.

Iron chelated in the soil by the above chelates becomes a part of a negatively charged anion which makes it less susceptible to conversion to an unavailable form and diminishes the tendency of the iron to be precipitated as an insoluble hydrated iron oxide. The absorption of chelated iron by the plant appears to be governed by those factors which control anion absorption. Iron chelates which exhibit the least tendency to bind hydroxyl ions and have the greatest stability, as measured by the stability constant, are the ones which show the least tendency to dissociate in rooting media (48).

Holmes & Brown (34) grew chlorosis-susceptible soybeans (PI-54619–5-1) on a calcareous Millville soil to which comparable rates of the five chelates were added. DTPA prevented chlorosis at approximately 120 p.p.m. of soil , whereas APCA was partially effective with as little as 3 p.p.m. It should be pointed out that although CDTA is known to bind iron more strongly than DTPA, it was ineffective in preventing chlorosis. However, Fe-CDTA was effective under the same conditions.

Metal-free chelating agents or iron chelates applied to soils are free to react with the mineral components of the soil. Some factors which determine

the end products of the reactions will be the relative stabilities of the metal chelates formed, the amount of chelating agent used, the chemical nature of the ions in the soil, and the physical accessibility of the ions in the soil to the chelating agent in order that the conversion to the metal chelate may take place.

An indication that iron may be made active through the presence of a chelating agent within the roots has been suggested by Weinstein *et al.* (100). Other workers (90) have grown plants in cultures containing Fe-EDTA in which the EDTA was labeled with a carbon or nitrogen isotope. The isotope was found in the plant tissue. Although EDTA appears to be resistant to normal metabolic processes, it is possible that the tagged atoms which were taken up were degradation products of the chelating agent.

Autoradiographs of soybeans grown on a calcareous Millville soil containing DTPA randomly labeled with C^{14} in the carboxyl group also showed the presence of radiocarbon in the plant tissue (34). Aqueous extracts of the leaves were passed through a Dowex 50 resin, and eluted with dilute ammonium hydroxide (48). Ninety per cent of the radioactivity was collected in those fractions where DTPA was known to appear, indicating the presence of the C^{14}-labeled chelating agent.

Soybeans treated with 1000 p.p.m. of APCA developed a pink color in the roots due to the formation and accumulation of Fe-APCA in the roots (34). More recently, Kroll *et al.* (48) treated bean plants by soil application with FeAPCA at high rates. The red chelate was visually discernible in the leaves 24 hr. after the application.

These observations indicate that iron chelates may be absorbed by the roots of plants and transported into the leaves. Seeberg *et al.* (73) found that iron from iron chelate was absorbed from the gastrointestinal tract of anemic rats and utilized for hemoglobin regeneration at the same rate as iron from ferrous sulfate. Applied intravenously the iron was not readily available to the body. It would appear that iron is released from the chelate in rats by some mechanism in the gastrointestinal tract before being absorbed; in the blood stream the metal ions remain attached to the chelate.

Although chelates look promising as a corrective for iron chlorosis where soil and plant factors are such that iron is not in a form which can be utilized by chlorosis-susceptible plants, these chemical agents have introduced several new problems which require clarification. The presence of the iron chelate at the root surface or in the plant tissue raises a question as to what processes are involved in removing the metal ion from the chelate and the incorporation of the metal into an enzymatic system. It is also of importance to understand the metabolic fate of the chelates in the plant since these compounds, if they persist in the plant fluids, may affect the mineral metabolism of the plant and other growth processes. The effect of APCA on the mineral metabolism of soybeans at a concentration of 3 to 10 p.p.m. in a calcareous soil suggests that this compound may have a favorable physiological effect in addition to its capacity to chelate iron (34).

Stewart & Leonard (83) suggest that chelates are probably the natural means by which iron is absorbed by plants. Differences in the capacity of plants to produce these naturally occurring materials could determine the capacity of a plant to take up iron. They believe it probable that the reason certain plants, such as azaleas, require acid soils is because of their incapacity to take up sufficient iron from soils having higher pH values. It would appear that the natural chelates in these roots cannot compete with the OH^- ion for iron in a high pH media. Depending on the kind and amount of naturally occurring chelates in the plant high concentrations of the heavy metals in the rooting medium may act competitively with iron. Calcium may also have this effect in some cases.

A continuous source of available iron appears necessary for the growth of all plants. The fact that iron chlorosis can be corrected in plants growing in the presence of the bicarbonate ion, excess heavy metals, or excess phosphate, or in calcareous soils by applying an available source of iron to the soil, provides strong evidence that iron deficiency is caused by a lack of available iron in the rooting medium.

It is apparent that the use of organic chelating agents has opened a wide field for research in controlling and explaining the pathways of mineral metabolism in plants. Whether or not chelation processes in plants can account for the marked differences in susceptibility of plant species and varieties to iron and copper deficiencies remains as a challenge for future research.

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PHOTOPERIODISM IN WOODY PLANTS[1]

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Although experiments with woody species were reported in the original classical papers of Garner & Allard (24), there have been relatively few subsequent papers on photoperiodic phenomena in trees, compared with the great mass of published material on the photoperiodic control of flowering in herbaceous plants. Nevertheless, such information as is at present available indicates that the proportion of species exhibiting well marked photoperiodic phenomena is at least as high among woody plants as for herbs. This neglect of woody plants is no doubt due to the opinion that work with tree species is necessarily slow and difficult, coupled with the view that photoperiodic effects on vegetative growth are less important or less interesting than those affecting flowering. As a consequence, our knowledge of the photoperiodic responses in trees is less complete than is the case for the flowering response in annual plants, particularly in relation to the mechanism of photoperiodism. Nevertheless, it is clear that any general theory of photoperiodism must be capable of embracing effects upon vegetative processes in woody species, as well as effects upon flowering.

Several instances are now known of ecotypic differentiation in a woody species with respect to photoperiodic response (59, 70), which can be correlated with differences in the daylength conditions occurring over the natural geographical distribution of the species. Such observations would seem to indicate that length of day is probably an important ecological factor for tree species. A closer study of photoperiodic phenomena in woody species is thus called for, both because of its broad implications for our understanding of the subject of photoperiodism as a whole, and because of its ecological importance, especially in relation to forestry practice.

The only previous reviews of this subject published in English appear to be those of Gevorkiantz & Roe (25) and Wareing (76), but an extensive discussion of certain aspects of this subject is given by Vegis (75). Most experiments with woody species so far reported have dealt with the effects of daylength on various aspects of dormancy, although a few experiments have also been carried out on the flowering responses of certain shrubby species. Vegetative effects can be conveniently studied in seedling trees, whereas flowering does not occur in many forest trees during a juvenile period which may last many years, by which time the trees have become too large for convenient experimentation.

Types of Photoperiodic Responses

Vegetative processes in woody species which have been shown to be affected by daylength include the duration of extension growth, internode

[1] The survey of the literature pertaining to this review was concluded in September, 1955.

extension, leaf growth in conifers, time of leaf abscission, duration of cambial activity, time of bud break, and seed germination.

Induction of dormancy.[2]—The effect of daylength upon extension growth has been investigated in over 60 species distributed among 35 genera and, in all except a few of these, well marked responses have been demonstrated (Table I). In all such cases, exposure to short-day conditions results in reduced extension growth, attributable to (*a*) earlier cessation of apical growth

TABLE I

SPECIES SHOWING PHOTOPERIODIC SENSITIVITY WITH RESPECT TO EXTENSION GROWTH

Species	Authority	Species	Authority
Abelia grandiflora	39	*Larix spp.*	8, 52, 83
Acacia melanoxylon	27	*Liquidambar styraciflua*	38
Acer spp.	24, 27, 33, 57	*Liriodendron tulipifera*	24, 38
Aesculus sp.	8	*Morus alba*	27
Ailanthus sp.	8	*Phellodendron amurense*	52
Aleurites spp.	22	*Picea abies*	72
Alnus incana	72	*Pinus* spp.	8, 27, 33, 38, 78
Betula spp.	27, 34, 72	*Populus* spp.	59, 70, 74
Calluna vulgaris	17	*Prunus persica*	41
Caragana sp.	8	*Pyrus ussuriensis*	52
Carpinus betulus	27	*Quercus* spp.	27, 33, 38, 43, 44, 83
Caryopteris Mastacanthus	17	*Ribes* spp.	18, 32
Catalpa speciosa	27	*Robinia pseudacacia*	38, 52
Citrus spp.	65	*Rosa* (Pernetiana)	17
Corylus spp.	8	*Salix* spp.	8, 52
Fagus spp.	27, 82	*Taxus baccata*	27
Fraxinus spp.	38	*Ulmus* sp.	8

which results in a reduced number of internodes, and (*b*) reduced internode extension. The reduction in total growth under short-days is often very striking, so that, for example, first year seedlings of *Robinia pseudacacia* and *Larix sibirica* attain a maximum height of only 3 to 5 cm. if maintained under short-days from germination (52). In species with a monopodial habit the onset of dormancy under short-days is marked by the formation of a terminal resting bud, and in those with a sympodial habit it involves abscission of the shoot apex. It appears that short-day treatment generally induces a state of winter dormancy in resting buds, which (except in *Fagus sylvatica*)

[2] The author prefers to follow the well-established usage of the general term "dormancy," rather than to adopt the terms "quiescence" and "rest," as suggested by Samish (64). A distinction is made, however, between "summer" and "winter dormancy" as defined by Doorenbos (20).

can be broken by chilling. The reduction in internode length under short-days may be so great that the seedlings assume a rosette habit (70).

Under long-days the onset of dormancy is delayed, and with many species when the daylength exceeds a certain "critical" duration, growth may be maintained continuously for at least 18 months under favourable temperature conditions, e.g., in *Liriodendron tulipifera*, *R. pseudacacia* (24, 38). At daylengths below this critical value the duration of the growing period increases as the length of the daily photoperiod decreases (52). In other species there appears to be no critical daylength above which growth can be maintained indefinitely, since even under continuous illumination seedlings of *Pinus silvestris*, *Acer pseudoplatanus* (78), *Phellodendron amurense* (27), etc. form terminal resting buds after a certain period of growth. Although a greater height is always attained under long-day than short-day conditions, maximum height is not always obtained under continuous illumination (52, 78). In species showing no critical daylength, the difference between the long-day and short-day types of response is thus quantitative rather than qualitative.

In considering the effect of daylength on the duration of growth, it must be recognised that this is only one aspect of the general problem of the control of extension growth. Apart from the influence of external environmental factors such as mineral nutrition, temperature, and daylength, in many species the duration of extension growth is apparently also affected by certain "ageing" processes of an endogenous nature (13).[3] Thus, even under constant photoperiodic conditions, first year seedlings of *P. silvestris* will cease extension growth after a certain period of time (78), apparently as a result of this internal ageing effect. The over-all period of growth in any given case will thus be determined by the interaction between the endogenous ageing process and external factors, such as daylength. In some species, the daylength factor may, under certain conditions, become overriding. For example, in species which exhibit a critical daylength, the photoperiodic factor over-rides the endogenous ageing when the critical daylength is exceeded. In species showing no critical daylength, however, the photoperiodic factor has only a modifying influence and apparently never becomes overriding. In such species, growth is determined primarily by the endogenous ageing process and is only modified by environmental factors, including daylength.

When seedlings of species which normally grow by rapid "flushes" of growth, e.g., *Quercus* spp., are maintained under long-days or continuous illumination, growth is not continuous but occurs as a series of such flushes (43, 44, 83). Thus, Leman (44) found that seedlings of *Q. suber* maintained under continuous illumination made eight successive flushes in a single

[3] This endogenous ageing process is regarded by Bünning (13) as being attributable to an "endogenous yearly rhythm." The present author, however, prefers to avoid the use of the term "rhythm," since it seems to carry certain connotations which are open to question.

season. This species will form several successive flushes in this way even under natural long-day conditions, whereas other species, e.g., *Pinus* spp. which normally form only one flush of growth in a single season, can be induced to behave like *Quercus* under abnormally long photoperiods or continuous illumination (8, 38,45,80). This subject is dealt with in more detail below.

Among species showing photoperiodic sensitivity, there appears to be no exception to the rule that dormancy is hastened by short-days and delayed by long-days. Although it was originally reported by Garner & Allard (24) that apple made greater growth under short-day than long-day treatment, this report was based upon an experiment in which the long-day "control" consisted of a single replicate, and subsequent observations (26, 32) have indicated that photoperiodic conditions have little or no effect on the duration of extension growth in various varieties of apple. Among related species, *Sorbus aucuparia* shows little response to daylength (68), whereas *Pyrus ussuriensis* shows marked responses (52). Other species in which daylength is reported to have no effect on extension growth include *Syringa vulgaris* (17), and certain species of *Fraxinus* and *Rosa* (68) although other species of the two latter genera show responses (Table I).

Leaf abscission.—Leaf abscission is affected by daylength conditions in certain deciduous species, the normal time of leaf-fall being delayed when natural daylength conditions are extended by supplementary illumination, or hastened by short-day treatment (24, 38, 49, 57). Thus, Garner & Allard (24) observed that seedlings of *Rhus glabra* and *Liriodendron tuliperifera*, grown in a greenhouse, dropped their leaves at the normal time under natural autumn daylength conditions but retained them throughout the winter under long-days.

Similar results were obtained by Kramer (38) for certain species, but he found that seedlings of *Liquidambar styraciflua* and *Quercus alba* retained their leaves even under natural short-day conditions in the greenhouse, although corresponding seedlings maintained outdoors dropped their leaves at the normal time. Similarly, seedlings of *Betula pubescens*, *R. pseudacacia*, *Acer pseudoplatanus* have been found to retain their leaves for long periods under short-days when maintained under warm conditions in a greenhouse (83). Conversely, natural leaf-fall in the autumn cannot be delayed indefinitely by long-days in the case of trees growing in the open in the vicinity of street lights (49). Thus, it is clear that temperature exerts an important modifying influence on the photoperiodic control of leaf abscission, and in certain species lowered temperatures in the autumn may be more important in determining the time of leaf-fall than reduced daylength. Olmsted (57) found that the age of the leaf is another factor modifying the photoperiodic control of leaf abscission in *Acer saccharum.*

Cambial activity.—Another type of response is seen in the effect of photoperiodic conditions on cambial activity. In many woody species the maintenance of cambial activity is apparently dependent upon continued ex-

tension growth, and when the latter ceases, cambial activity ceases shortly after (62). This dependence of cambial activity upon extension growth is believed to arise from the fact that cell division in the cambium is stimulated by auxin produced in the region of the shoot apex, and that auxin production is highest in actively growing shoots (2, 69). In species to which this applies, any direct effect of daylength conditions on extension growth may be expected to have a secondary effect on the duration of cambial activity. In other species, however, e.g., *P. silvestris*, *Quercus robur*, extension growth ceases very early in the season, whereas cambial activity continues for several months longer (14, 87). In such species, maintenance of cambial activity is evidently not dependent upon active extension growth. Studies with second year seedlings of *P. silvestris* showed that the duration of cambial activity in this species is affected by photoperiodic conditions, independent of any effect of the latter on extension growth (80). Thus, exposure of the seedlings to experimentally shortened days hastened the cessation of cambial activity, while extension of the natural photoperiod by low-intensity supplementary illumination prolonged the duration of cambial activity.

The effect of daylength conditions on cambial activity has also been investigated in *R. pseudacacia* by Mollart (51). Seedlings were first exposed for three weeks to short-day conditions, which resulted in the cessation of extension growth and abscission of the shoot apices (as this is a species with a sympodial habit). Half the plants were then transferred back to long-day conditions, while the remainder were continued under short-days. The buds in both series of plants remained dormant. When the plants were sectioned 6 to 10 weeks later, it was found that the cambium was active in a a high porportion of the long-day plants, whereas in all the short-day plants it was dormant. Thus, even in seedlings in which extension growth had been stopped, photoperiodic conditions affect cambial activity. The new wood formed under long-days after extension growth had ceased, was of the summerwood type, and summerwood vessels appeared to arise from the leaf-traces of the mature leaves, suggesting that a cambial stimulus arises in such leaves under long-days, but not under short-days.

Breaking of dormancy.—Since short-day treatment brings about the cessation of extension growth and the formation of resting buds, the question arises whether dormant buds can be induced to resume growth by exposure to long-day conditions. In considering this problem it is important to make a distinction (*a*) between buds which are in a state of summer dormancy and those in winter dormancy and (*b*) between chilled and unchilled winter resting buds. Buds in a state of summer dormancy are much more easily induced to break by long-day treatment than ones which are in the winter dormancy condition. For example, during the summer the buds of *P. silvestris* show marked meristematic activity (involving the laying down of the initials of the next season's shoot) until well into August, and are clearly still in a state of summer dormancy. At this time, transfer of the seedlings to continuous illumination rapidly brings about premature expansion of the buds, which

otherwise would not break until the following spring (80). When growth of the buds is complete, and they have entered a state of winter dormancy they will not respond to continuous illumination and can then apparently only be induced to resume growth after winter chilling. [By contrast Gustafson (28) found that unchilled buds of *Pinus resinosa* expanded on exposure to 16-hr. photoperiods in the spring.]

The behaviour of species, such as *Q. robur*, which normally produce two or more distinct flushes of growth during a single season is similar. In such species, the terminal buds are not in a state of deep dormancy during the periods of quiescence between successive flushes and there is no difficulty in inducing a further flush of growth by long-day treatment, so long as the buds are in the condition of summer dormancy. However, once winter resting buds have been formed, further growth cannot be induced even by continuous illumination (37, 44). Thus, in *P. silvestris* and *Q. robur* summer dormancy is readily broken by exposure to long-days, but winter dormancy is not.

In other species, however, the buds respond to long-day treatment at all times, regardless of the state of dormancy. Thus, Klebs (37) showed that resting buds of *Fagus sylvatica* can be induced to resume growth at any time, whether the seedlings are in the leafy or leafless condition, by exposure to continuous illumination. This was not recognised by Klebs as being a photoperiodic phenomenon, but has subsequently been shown to be so (81). He showed that in order to obtain renewal of growth in leafless seedlings of *F. sylvatica*, the buds themselves must be directly exposed to long-day conditions, and it appears that photoperiodic "perception" occurs in meristematic tissue of the rudimentary leaves and internodes (81). It is not known what proportion of other species resemble *F. sylvatica* in the capacity to respond to long-day treatment at any stage of bud development, but in a preliminary survey (83) it was found that the buds of *B. pubescens* and *Larix decidua* could be induced to break after 2 to 3 weeks exposure to continuous illumination. On the other hand, seedlings of *Acer pseudoplatanus* and *Robinia pseudacacia*, which had been rendered dormant by previous short-day treatment, could not be induced to resume growth by 8 to 10 weeks of continuous illumination.

It would appear, therefore, that there are rather marked differences between species in their capacity to respond to continuous illumination by breaking dormancy. Such differences, however, may be of a quantitative nature only, since Olmsted (57) obtained slow breaking of dormancy of unchilled seedlings of *A. saccharum* under long-day conditions, but even after four months of treatment only 64 per cent of the seedlings showed bud break, as compared with 35 per cent under short-days. Van der Veen (74) was also able to induce unchilled seedlings of *Populus robusta* to break dormancy by exposure to continuous illumination, but only after a delay of several months. On the other hand, Gulisashvili (27) tested the effect of continuous illumination on the breaking of dormancy of seedlings of a number of different woody species from various geographical regions in Russia and ob-

tained fairly rapid breaking of dormancy of unchilled buds in all species, except *P. silvestris*. Seedlings of *Acer trautvetteri* (45 days) and *Quercus iberica* (32 to 47 days) actually broke dormancy more rapidly than *Fagus orientalis* (45 days) and *Betula verrucosa* (45 days). These results are somewhat at variance with those of other authors cited above, but the discrepancies may be due to differences in the conditions of light intensity and temperature used (which were not stated), or to differences in the initial depth of dormancy of the buds. In view of its possible practical importance as a forcing technique in horticulture and breeding work, further information is required on this subject.

The interaction between photoperiodic effects and chilling requirements has not been investigated in detail. It appears that the buds of *F. sylvatica* show no response to chilling, since even after a prolonged period of storage at 0° to 5°C. the photoperiodic requirements for the breaking of dormancy remained unchanged (81). Buds of *B. pubescens* do respond to chilling, however, and after six weeks at 5°C. they will break not only under short-days but even in continuous darkness (84). Similarly the buds of species which do not readily respond to photoperiodic treatment when unchilled, e.g., *R. pseudacacia*, *A. pseudoplatanus* (81) and *Populus* spp. (59) readily break dormancy under all daylength conditions, and even in continuous darkness, after chilling. Breaking of chilled buds may occur more rapidly under long-day than short-day, however (27, 38). The substitution of a chilling requirement by long-day treatment has also been observed in dwarfed seedlings of peach derived from unchilled embryos (41). Exposure to long-day conditions removed the dwarfed habit, especially at higher temperatures (22° to 24°C.)

Flowering response.—Reports of experiments on the flowering responses of woody species to photoperiodic conditions are very few in number. Allard (1) investigated the flowering responses of four woody species. Poinsettia (*Euphorbia pulcherrima*) and Bougainvillea (*Bougainvillea glabra*) were found to behave as short-day plants, whereas *Hibiscus syriacus* was a long-day plant. *Malvaviscus conzattii* appeared to be a day-neutral species.

Chouard (17, 18) found that the normal flowering of *Calluna vulgaris*, *Caryopteris mastacanthus*, *Ribes rubrum*, and *Rosa* (Pernetiana) under natural long-day conditions is completely suppressed under short-days. On the other hand, in *Syringa vulgaris* and cultivated cherry, flower-initiation (which occurs within the resting buds as they are developing in June and July) was not affected by daylength conditions.

The requirements for floral initiation in *Cestrum nocturnum* are complex (63). Flowering occurs if the plants receive long-days followed by short-days. Continuous short-days or long-days, or short-days preceding long-days, are ineffective.

Sceglova & Leisle (66) have shown that long-day conditions are necessary for the initiation of flowers in *Ulmus glabra*. It was shown that experimental exposure of individual shoots of a large tree to short-day conditions during the period June 19 to July 15 suppressed the formation of flower initials in

the treated shoots, whereas control shoots exposed to natural long-day conditions during this period readily initiated flowers (within the differentiating resting buds).

Photoperiodic treatment apparently has little effect on flower-initiation in azalea (9, 61) and apple (26, 32). Reports on the effect of photoperiodic conditions on flower initiation in the gardenia are somewhat conflicting (4, 36, 60).

There are a few reports of the effect of daylength conditions on the duration of the juvenile period in woody species. Thus, Langlet (42) observed that races of *P. silvestris* from Northern Sweden grown at lower latitudes commenced to form female cones at the age of three years, as compared with the normal age of five to seven years. He regards this as a possible photoperiodic effect arising from the shorter daylengths in the more southern station. Moshkov (55) observed that seedlings of *Ribes nigrum* and *Salix phylicifolia* flowered in the first year of growth under continuous illumination, whereas under natural daylength conditions this did not occur until the third year. Nikitin (56) exposed seedlings of oak to continuous illumination during the first five months following germination. The seedlings were then transferred to the open under natural daylength conditions. They later flowered and produced fruits in the eighth year of growth, whereas usually the duration of the juvenile period is 40 to 60 years. The occurrence of a long-term photoperiodic after effect in a woody species has also been reported by Scepotjev (67), who studied the effect of short-day treatment on leaf-shape in *Morus alba*. Seedlings exposed to short-day treatment in the first year of growth were found, seven years later, to show leaf characters differing significantly from those of untreated seedlings with regard to dry weight and degree of incision, the treated trees having more juvenile leaves than the controls. In view of their possible importance for the breeding of forest trees, these reports of long-term after-effects require confirmation.

The Locus of Photoperiodic Perception in Relation to Dormancy

It is well known that photoperiodic induction of flowering in herbaceous species is determined primarily by the daylength conditions to which the partially- or fully-expanded leaves are exposed, and that there appears to be little or no direct effect of photoperiodic conditions upon the meristematic tissues of the shoot apex (15, 30, 54). This is in marked contrast to the observation that long-day conditions may stimulate the breaking of resting buds of leafless seedlings of *F. sylvatica*, and that the site of perception is the meristematic tissue of the bud itself (81). Moreover, the effect of daylength upon internode extension in *P. silvestris* was shown to be a direct one upon the meristematic tissue of the young extending internodes (79).

These observations raise the following questions: (*a*) Are the fully-expanded leaves also sensitive to photoperiodic conditions in woody species? If so, how far is the response of the buds affected by the presence of mature

leaves in dormant seedlings which still retain mature leaves? (*b*) What is the locus of photoperiodic perception in actively growing seedlings for the induction of dormancy by short-days? Is the actively growing shoot apex directly sensitive to daylength conditions, or does the stimulus for the formation of resting buds arise in the mature leaves under short-days?

Information on the first of these questions was provided by experiments with seedlings of *B. pubescens* which had been induced to become dormant by previous short-day treatment, but which had been allowed to retain the mature leaves (83). The leaves and the buds were exposed independently to long-day or short-day conditions. It was found that in order to obtain breaking of dormancy by long-day treatment, both buds and leaves must be exposed to long-days. Thus, whereas exposure of the buds alone results in bud-break in leafless seedlings, when the buds are exposed to long-day and the leaves simultaneously to short-day, breaking is inhibited. Evidently, leaves maintained under short-day have an inhibitory effect on bud growth. On the other hand, the removal of this inhibitory effect by exposing the leaves to long-day is not, in itself, sufficient to induce bud-break, which also requires direct exposure of the buds to long-days. These observations were interpreted to indicate that some inhibitory effect is transmitted from the leaves to the buds under short-day conditions, but that there is no transmission of a growth promoting stimulus from the leaves under long-days.

The conditions necessary for the production of secondary flushes in *Q. robur* were found to be similar to those necessary for the renewal of growth in *B. pubescens*, viz., both the mature leaves and the shoot apex must be exposed to long-day conditions (83).

In order to answer the second question raised above, similar experiments were carried out with actively growing seedlings of three species, in which the shoot apex and mature leaves were exposed independently to long-day or short-day conditions. In the case of *B. pubescens* it was found that the formation of resting buds is determined primarily by the daylength conditions to which the shoot apex is directly exposed, regardless of whether the leaves are maintained under long-day or short-day conditions. Thus, in this species, both the dormant resting buds and the active shoot apex show marked photoperiodic sensitivity. When the mature leaves are maintained under short-day conditions, they have an inhibitory effect on the breaking of dormancy of the buds, but this inhibitory effect is not sufficient to suppress growth of an active apex maintained under long-days. The situation in the other two species tested, *R. pseudacacia* and *A. pseudoplatanus*, is reversed, however, since in these species it was found that the induction of dormancy is determined entirely by the conditions to which the mature leaves are exposed, and that it is immaterial under what photoperiodic conditions the shoot apex is maintained (83). In these two species, therefore, the situation with regard to the induction of dormancy is comparable with that in herbaceous species with respect to flower induction. Moreover, it is probable that the difference between *B. pubescens* and herbaceous species with respect to

the photoperiodic sensitivity of the shoot apex is one of degree only, since recent experiments have indicated that in *Xanthium* the partially expanded leaves are more sensitive than mature leaves (40). Since the buds of leafless seedlings of *R. pseudacacia* and *A. pseudoplatanus* are not easily induced to break dormancy when exposed to continuous illumination, it would seem that photoperiodic sensitivity is slight or absent in both resting buds and active apices of these species, and that the difference in the behaviour of these species as compared with that of *F. sylvatica* and *B. pubescens* originates primarily from this fact.

The Partial Processes of Photoperiodism in Woody Species

It is possible to recognise a number of distinct partial processes involved in the photoperiodic induction of flowering. [Since this subject has recently been discussed in detail by Liverman (47), it is necessary to give only a brief summary here.] It is well established that in short-day plants a primary light reaction is involved in which the light intensity requirements are relatively high and which is probably photosynthesis. As a result of this light reaction, photosynthates necessary for the processes in the following dark period are formed. It has been further established that the absolute length of the daily dark period plays a determinative role in the induction of flowering, which in short-day plants depends upon exposure to cycles which include a certain minimum period of unbroken darkness (critical dark period). In long-day plants, on the other hand, flowering in inhibited when the cycles include dark periods in excess of the critical duration. In order to effective, an adequate dark period must be preceded by the period of high intensity illumination. The effect of the dark period can be nullified in both short-day and long-day plants if it is interrupted by a short light break or by quite low intensity continuous illumination. Thus, a secondary, low intensity photo reaction is also involved, which is inhibitory to flowering in short-day plants and promotive in long-day plants. Less information is available regarding the corresponding phenomena in woody species, but sufficient is known to make possible a comparison with the flowering effects in herbaceous species. In this connection, special interest attaches to the occurrence of photoperiodic responses in resting buds and seeds, since presumably no appreciable photosynthesis occurs in such tissues, and the existence of a high intensity light requirement is thus not to be expected in these organs.

If we consider, first, the induction of dormancy in actively growing leafy seedlings of woody species, it is found that there are certain analogies with the induction of flowering in short-day plants. Thus, in certain woody species there is a well marked critical daylength requirement for the induction of dormancy. This type of response would appear to be comparable to the unlimited vegetative growth resulting when short-day species are maintained under long-day conditions, and the induction of flowering when the daylength falls below a certain critical value. The requirement for a high inten-

sity light reaction for the induction of dormancy in leafy seedlings of woody species has not yet been demonstrated. Neither has it been ascertained whether the response of such seedlings is determined primarily by the duration of the daily dark period. The observation that a light-break or continuous low intensity illumination given during the dark period delays the onset of dormancy under short-days (32, 78) suggests, however, that the same secondary light reaction is involved as in herbaceous species, and that certain dark processes are inhibited in a similar manner in both types of plants. Thus, although direct experimental evidence is slight, such information as is at present available is compatible with the view that the same types of partial processes are involved in leafy seedlings of woody species as in herbaceous plants. This conclusion is also in agreement with the observation that the leaves of woody plants are sensitive to photoperiodic conditions and play a determinative role in the photoperiodic behaviour of certain species.

On the other hand, a study of the responses of buds of *F. sylvatica* (81) gave no evidence of any light reaction corresponding to the primary, high intensity photoreaction of herbaceous species, since the light requirement of the buds is fully saturated at intensities of about 1000 lux. From observations on the transmissive properties of the bud scales it was estimated that light of this intensity at the bud surface corresponds to an intensity of approximately seven lux within the meristematic tissue of the bud, so that the possibility of any appreciable photosynthesis being involved in the photoperiodic reaction of the bud seems to be excluded. The renewal of growth of the buds seems to be determined primarily by the duration of the dark period, since breaking occurs when they are exposed to short photoperiods, provided that the associated dark periods are also short. There appears to be no sharply defined critical dark period in buds of *F. sylvatica*, since an increasing percentage of bud break is obtained as the day length is increased from 12 hr. to continuous illumination. Interruption of long dark periods by light breaks results in bud growth, i.e., the light breaks produce the long-day type of response, as in herbaceous species, although it is possible that greater quantities of energy are required in buds than in leafy plants.

When the scales were removed from the buds so as to expose the young leaf initials, continuous illumination brought about rapid leaf expansion and internode extension as compared with plants in continuous darkness. It would appear, therefore, that perception of the light stimulus occurs in the meristematic tissue of the rudimentary leaves and internodes. On the other hand, the de-scaled buds kept in darkness also expanded after considerable delay, which is never the case with intact buds. It is thus possible that the bud-scales have an inhibitory effect which the meristematic tissue is able to overcome under long-day conditions.

The observation that both resting buds and active shoot apices of birch (*B. pubescens*) show photoperiodic sensitivity suggested that possibly the

"seeds" (achenes) of this species might show photoperiodic effects in relation to germination, and this was found to be the case (6). The photoperiodic responses of the seeds have been investigated in some detail by Black & Wareing (7), since they provide the same special features of interest as buds, in that the photoperiodic effects occur in meristematic, nonphotosynthetic tissue, and they constitute more convenient material for photoperiodic experiments than dormant seedlings.

The photoperiodic behaviour of birch seed is markedly modified by temperature. At 15°C. germination of the unchilled seed is markedly affected by daylength conditions, a germination of approximately 95 per cent being obtained with 20 hr. photoperiods, whereas with daylengths of 8 hr. or less the germination is only about 30 per cent. Maximum germination is obtained after exposure to 8 cycles. This response is determined primarily by the duration of the daily dark periods; a high germination occurs with short photoperiods, provided that the associated dark periods are short also. As in beech buds, however, this is no clearly defined critical dark period, since a gradually increasing percentage germination is obtained as the daylength is raised to continuous illumination. A single, low intensity photo-reaction appears to be involved, red light of the intensity 1000 ergs/cm^2/sec. being well above the saturation level. At 20°C., the unchilled seeds still require light but the photoperiodic nature of the response is lost, since a single light exposure of 15 min. duration gives over 50 per cent germination. Thus, at the higher temperature, birch seed behaves like other light sensitive seeds, e.g., lettuce, in that a single light exposure is sufficient to produce a high percentage germination. The promotive effect of a single exposure to red light can be completely nullified if followed by exposure to infrared radiation. Evidently the same photoreceptive pigment system is involved as has been postulated by Borthwick and his co-workers for lettuce seed (10).

If birch embryos are excised from the endosperm and pericarp and planted on moist filter paper, then they readily germinate in complete darkness at 15°C. It would appear that the endosperm or the pericarp has an inhibitory effect on the germination of the embryo, which it is able to overcome when exposed to light. After a period of three to four weeks of chilling at 0° to 5°C. in moist storage, birch seed no longer requires light but will germinate if transferred to warm conditions in complete darkness. In this respect the behaviour of the seed corresponds closely to that of the resting buds of this species, which will only break in response to long-days when unchilled, but will grow in complete darkness after a period of chilling.

It is of interest to compare and contrast these effects in buds and seeds with the phenomena associated with the photoperiodic control of flowering. The absence of a high intensity light reaction in buds and seeds is not surprising, since this reaction is believed to be photosynthesis and hence is to be expected to occur only in green tissue. Since a period of high intensity illumination is necessary before the dark processes can occur in short-day plants the absence of a high intensity photoreaction in buds and seeds raises the question whether corresponding dark processes occur in these

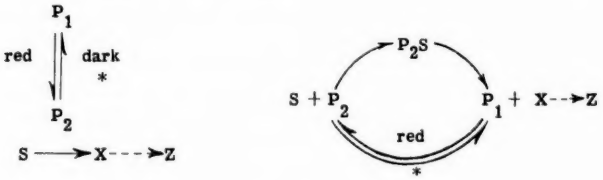
organs. If the requirement for a high intensity photoreaction in the flowering response is primarily for respiratory substrates (47), however, the absence of appreciable photosynthesis in seeds and buds presents no special difficulty, since the level of reserve materials in these organs is, no doubt, adequate to meet respiratory requirements. The observation that resting buds may be formed in complete darkness in the aquatic plant, *Hydrocharis morsus ranae*, provided sugar is supplied in the external medium (75), is of interest in this connection.

Since growth of unchilled buds and seeds is promoted by light, the effect of darkness must be either (*a*) neutral or (*b*) actively inhibitory. One of the pieces of evidence for active dark processes in the control of flowering is provided by the fact that increased temperature during the dark period enhances the effectiveness of the latter (30, 50), indicating a positive temperature coefficient for certain dark processes. Similarly, if there is an actively inhibitory dark process in birch seeds, it is to be expected that an increase in temperature during the dark period would result in a low germination percentage, whereas, in fact, an increased germination is obtained by this treatment. It would appear, therefore, that there is no active dark process in birch seeds comparable to that occurring in the leaves of long-day and short-day herbaceous plants. On the other hand, the observation that excised birch embryos have no light requirement for germination suggests that in the intact seeds germination is inhibited by the pericarp or endosperm, but presumably the inhibition from such tissues is not dependent upon active metabolic processes (see below) and hence does not show a marked positive temperature coefficient. Such inhibition, moreover, will not be limited to the dark period. Evidently light enables the embryo to overcome this inhibition, and it seems probable that photoreception is located in the living tissue of the embryo. The position thus appears to be closely comparable to that in buds of *F. sylvatica*. On this view, the photoperiodic responses of the intact seeds and buds are to be interpreted primarily in terms of the promotive effect of light, the effect of the dark period being a passive one.

At 20°C. the responses of birch seed resemble those of lettuce in that (*a*) a single light exposure is sufficient to give a high germination percentage, and (*b*) red and infrared radiation have mutually antagonistic effects upon germination. The hypotheses put foward by Borthwick and his co-workers (10, 11, 71a) for lettuce and *Lepidium* seed could, therefore, be applied equally to explain the behaviour of birch seed at 20°C., but they do not account for the photoperiodic nature of germination response at 15°C., which may be explained in terms of the following hypothesis (5). The basic postulates are as follows: (*a*) As in lettuce seed, photoperception involves a pigment which can exist in one of two mutually interconvertible forms P_1 and P_2. Form P_1 is transformed by red radiation into the form P_2, which reverts spontaneously to P_1 in darkness, or more rapidly under the influence of infrared radiation. (*b*) The pigment form P_2 catalyses the production of some substance X from a substance S. By a series of further steps, X gives rise to a substance Z.

Germination is dependent upon the attainment of a certain threshold concentration of Z.

The hypothesis is shown schematically in I. It will be seen that in this hypothesis not attempt is made to indicate the nature of the transformations involved in the interconversion of P_1 and P_1, or how P_2 catalyses the reactions S→X. It is possible that there is a direct reaction between P_2 and S, as postulated by Toole *et al.* (71)[4] for *Lepidium* seed, and if P_1 is regenerated from P_2 during this reaction, the over-all process would be as indicated in scheme II, which incorporates certain features of the hypothesis of Liverman & Bonner (48). At 20°C., sufficient Z to cause germination is evidently formed following a single light exposure, but at 15°C. repeated light expo-



* Refers to infrared.

sures are necessary. Apparently accumulation of Z over successive cycles is required before the necessary level is attained at 15°C. A high germination can still be obtained if the higher temperature treatment is restricted to a period of dark storage following a single light exposure at 15°C. Although the effect of the higher temperature is thus not primarily upon the photoreaction, nevertheless the increased temperature must affect certain dark reactions initiated by the light exposure, since it must be given following (and not preceding) the light exposure. It seems unlikely that the higher temperature affects the reaction between S and P_2, since the application of the higher temperature may be delayed for two days following the light exposure, by which time any free P_2 will presumably have reverted to the form P_1. It would seem, therefore, that it is the slow rate of one of the later stages which is affected by the reduced temperature at 15°C., and that repeated cycles are necessary for the accumulation of Z to a certain threshold value. Now, with repeated cycles at 15°C., the germination percentage falls progressively as the duration of the dark period increases (with a constant length of photoperiod) and when it exceeds about 24 hr. there is no appreciable germination. Thus at 15°C., there is no summation with successive cycles when the dark period exceeds a certain duration, presumably because some product decays progressively as the length of the dark period is increased. Thus, we may postulate that during and immediately following a light exposure, some sub-

[4] For a statement and discussion of this hypothesis, see the paper by Toole *et al.* on "Physiology of Germination" in the present volume.

stance is formed which is relatively labile, and which is, therefore, not accumulated over successive cycles when the intervening dark periods are too long. Such a hypothesis would explain why the response is determined primarily by the duration of the dark period. Apparently the temperature coefficient for the breakdown of this substance is not as high as for its production, so that reduction from 20° to 15°C. lowers the rate of production more than the rate of breakdown. On the foregoing hypothesis, it is possible to explain photoperiodic responses in buds and seeds in terms of the promotive effect of the low intensity photoreaction, without the need to postulate any active dark process.

It is noteworthy that several of the photoperiodic effects produced in woody species by short-day treatment, viz., cessation of apical growth, reduced internode extension, leaf abscission, and cessation of cambial activity, are all conditions associated with low auxin levels in the plant. This fact suggested that the photoperiodic responses of woody species are determined primarily by the effect of daylength conditions upon the auxin level within the plant, short-days producing low and long-days high auxin level (77, 80). There is, indeed, evidence that light is necessary for auxin production in the green plant (3), and several instances have been reported (16, 46) of greater yields of diffusible auxin from leaves under long-days than under short-day conditions. In such instances, however, the light intensities used have been relatively high, and the effect upon auxin production may have been exerted partly through the greater quantity of photosynthates available under long-day conditions. Nevertheless, there is evidence that the low intensity photoreaction interacts with auxin in the plant, red light being found to enhance auxin controlled growth in certain tissues (48). This is consistent with the fact that the long-day responses observed in woody species, which are also characteristic of high auxin levels, may be produced when quite low intensities are used to supplement short photoperiods of daylight. The observation that a cambial stimulus is produced in mature leaves of *Robinia* by low intensity supplementary illumination would seem to be of special interest in this connection.

Although it is possible to interpret effects in buds and seeds solely in terms of the growth promoting effects of light, nevertheless, there is evidence that when leaves are present, they have an inhibitory effect on bud growth when maintained under short-day conditions. Thus, it was shown that there is a direct promotive influence of light on the bud under long-days which is localised in its effect, and an inhibitory influence which is transmitted from the leaves when the latter are maintained under short-days. It appears, therefore, that a positive dark reaction which is inhibitory to growth occurs in the leaves of woody species under short-days. We are thus led to the conclusion that there is some actively inhibitory process in the leaves under long dark periods, whereas in seeds the role of the dark periods is passive.

Dormancy in seeds, buds, and other organs has been attributed to the presence of growth inhibitors. The occurrence of germination inhibitors in seeds is well known (23), and their possible role in bud dormancy has been

discussed by Samish (64). It is tempting to postulate, therefore, that the cessation of growth and the onset of dormancy in leafy seedlings under short-day conditions is attributable to the production of a growth inhibitor in the leaves and its accumulation in the resting buds and seeds during development. The inhibitory effect of the pericarp and endosperm in birch seed may be due to the presence of an inhibitor, since a water soluble fraction has been extracted from the seeds which is capable of inhibiting excised embryos (5). If this is the case, the embryo is evidently enabled to overcome this inhibition by light. If the inhibitor is derived from the mother plant and is not actively produced in the detached seed, then the absence of an actively inhibitory dark process in seeds is understandable. On the other hand, production of an inhibitor by leaf tissue would be an active process, with a positive temperature coefficient. Possibly the occurrence of a well defined critical dark period in leafy plants, and its absence in seeds, is to be ascribed to the active production of inhibitor in the former, and the derivation of a fixed amount of inhibitor from the mother plant in the latter (7).

It is of interest to consider the implications of these results with woody species for the general theory of photoperiodism. It has been shown that the responses of birch seeds appear to be determined by an interaction between the promotive effects of light and an inhibition of germination due to the endosperm or pericarp. In leafy seedlings of woody plants the inhibitory effect on growth arising from long dark periods is clearly apparent, but the question arises whether low intensity light has an actively promotive effect on growth in leafy plants, as well as in buds and seeds. Now, it is generally assumed, in relation to flowering of herbaceous species, that the effect of low intensity light is simply to nullify the effect of the dark period (apparently by converting the light activated pigment system into the form P_2), and that there is no accumulation of some product arising from the low intensity reaction, which is inhibitory to flowering in short-day plants and promotive in long-day plants. The responses of first year seedlings of *P. silvestris*, however, appear to be determined by an interaction between (*a*) a growth promoting effect of the light period which is carried over into the early part of the following dark period, and (*b*) a growth inhibiting system which becomes operative during the later part of the dark period (78). (At the time that this hypothesis was put forward it appeared that the promotive effect was due to the high intensity photoreaction, but the evidence for this was not conclusive.) Moreover, there is evidence from studies of certain short-day herbaceous species that the low intensity photoreaction may result in the accumulation of some flower-inhibiting substance. Thus, in Biloxi soybean, flowering will not occur when the photoperiod exceeds a certain limiting duration, even when associated with long dark periods (29). On these and other grounds, it was postulated (82, 85) that the low intensity photoreaction results in the active production of a substance "X" which inhibits flowering in short-day plants. It is held that during the main photoperiod not only are photosynthates formed, but the secondary, low intensity photoreaction

proceeds concurrently and leads to the gradual accumulation of X. When the length of the photoperiod exceeds the limiting duration, X attains a level which is completely inhibitory to flowering. Such a hypothesis is not difficult to reconcile with that of Borthwick *et al.*, if it is postulated that the continued maintenance of the pigment system in the form P_2 during a prolonged light exposure results in the gradual accumulation of X. Thus it would appear that not only in light sensitive seeds and leafy seedlings of woody plants, but also in typical herbaceous short-day plants, the low intensity photoreaction results in the active accumulation of some product X which is presumably identical in all types of plant tissue. In this way it is possible to relate the phenomena in woody species to those associated with the flowering responses of short-day plants.

On the other hand, the photoperiodic phenomena in woody species must ultimately be related to the general problem of dormancy. At the present time it is not possible to visualize how the phenomena in these two branches of developmental physiology are related, but this would seem to be a very promising field of study and may possibly offer a more direct means of relating photoperiodism to general metabolism than a study of flowering responses.

Ecological and Practical Importance

The recognition that various growth processes in woody plants are affected by the daylength regime raises the question as to how far such processes are normally controlled by seasonal variations in day length under natural conditions. It need hardly be pointed out, however, that the demonstration of photoperiodic responses in relation to experimentally controlled daylength conditions does not imply that such effects will necessarily occur in nature. Clearly the term "control" requires definition, for any given growth process is subject to a variety of modifying influences of both "internal" and "external" origin. Under certain conditions, however, one or more of these factors may become limiting, and in the present context we may speak of "photoperiodic control" when the daylength conditions are changed from limiting to nonlimiting, or vice versa, so that initiation or cessation of some growth process occurs. Thus, the essential requirement for photoperiodic control is that initiation or cessation of some growth activity shall occur in response to a seasonal change in natural daylength conditions.

Photoperiodic control of the cessation of extension growth under natural conditions has been directly demonstrated for *R. pseudacacia* in which seedlings normally become dormant when the natural photoperiod falls to about 12 hr. in September. If, however, the seedlings are exposed to supplementary illumination to extend the natural daylength at this time, the growing period is prolonged beyond its normal duration, until ultimately temperature conditions become limiting (38). Similar effects have been demonstrated for *Populus trichocarpa* (59). By analogy, it seems probable that the cessation of extension growth is photoperiodically controlled in

other species, e.g., *Larix, Populus, Salix, Betula*, etc., which continue growth until well into the autumn, and which are known to show marked responses to short-day conditions.

In other woody species, e.g., older trees of *Alnus glutinosa, Acer pseudoplatanus, Platanus orientalis*, etc. (76), extension growth ceases in June or July before there has been any appreciable reduction in the natural length of day. In such cases the cessation of extension growth at this time cannot be due to the fact that daylength conditions have become limiting and it seems probable that the duration of extension growth is here controlled endogenously. A number of the species falling in this second category, i.e., those in which extension growth is not normally subject to photoperiodic control, nevertheless show marked responses when exposed experimentally to short-day conditions. Indeed, of the considerable number of species which have been found to show hastened dormancy under experimental short-day conditions, probably only a small proportion show extension growth actually subject to photoperiodic control in nature.

In many woody species the seasonal period of extension growth is much shorter in mature trees than in the seedling stages (76). For example, although seedlings of *R. pseudacacia* have a long growing period which extends into the autumn, in mature trees of this species extension growth of many shoots may have ceased by the end of July. Presumably, in older trees, other factors (such as competition between the various shoots for nutrients) bring about cessation of growth before daylength conditions become limiting. Thus, conclusions regarding mature trees cannot be arrived at by extrapolation from the photoperiodic behaviour of the seedlings.

It is not clear to what extent leaf abscission in the autumn is normally controlled by photoperiod, since reduced temperatures are also important and in certain species may be the primary controlling factor. Evidence has been obtained that the cessation of cambial activity may be controlled by photoperiodic conditions in certain species, although the initiation of cambial activity appears to be independent of seasonal changes in daylength (19).

In those species which show a chilling requirement, bud break is probably determined primarily by rising temperatures in the spring, since chilled buds will break independent of day length when transferred to warm conditions. In the case of *F. sylvatica*, however, chilling appears to be without effect, and it appears probable that increasing daylength may control the time of bud break under certain climatic conditions, although, in England, by the time spring temperatures have risen sufficiently to permit growth, daylength is no longer a limiting factor (81). It seems possible that photoperiodic control of bud break may be operative in certain tropical woody species, in which chilling requirements are presumably absent.

There is little doubt that the time of flowering in certain shrubby species, e.g., Poinsettia (*E. pulcherrima*) is controlled by the onset of short-day conditions in the autumn (1). Sufficient information is not yet available, however, to make it possible to determine to what extent daylength con-

trols the time of flower initiation in temperate forest trees. The observation that flower initiation in *Ulmus glabra* can occur only in long-day conditions does not necessarily imply that the time of initiation under natural conditions is photoperiodically determined. The actual time of flower initiation, viz., the end of June and early July, is not associated with any marked seasonal changes in daylength, and the formation of flower primordia is probably determined by some internal ontogenetic mechanism. Thus, although in this species flower initiation cannot occur under short-days, temperature conditions normally restrict growth to a period of the year when daylength conditions are not limiting for reproductive development. Similar arguments apply to a number of other tree species, e.g., cherry (17) which initiates flower primordia (within resting buds) at this time of the year.

In herbaceous species it is generally assumed that the biological significance of photoperiodism lies in the control of flowering and that vegetative responses are secondary. It does not follow that this is true also for woody species, however, in which the primary significance of photoperiodism may concern dormancy phenomena. There is, indeed, considerable evidence that dormancy phenomena are of primary importance in the adaptation of many woody species to their natural habitats. In temperate regions, the duration of the frost free period is a major factor of the environment and limits the duration of the period of active growth. Since trees are more resistant to frost damage in the dormant condition than in the actively growing state, it is clear that the time of onset of dormancy may play a critical role in determining whether a given species can survive under certain climatic conditions. In those species in which dormancy phenomena are markedly affected by the photoperiodic conditions, it is clear that the latter constitute an important factor of the environment to which the species must be adapted. A knowledge of this fact is obviously of importance to the forester, particularly in relation to the introduction of exotic species into new regions (53, 58, 76). Some species from lower latitudes when introduced to regions of higher latitude tend, because of the longer natural photoperiods, to continue growing longer than in their region of origin, and thereby become liable to damage by autumn frosts (52, 68). On the other hand, species or races indigenous to higher latitudes must be expected to show reduced growth in lower latitudes. These considerations apply not only to species in which the duration of extension growth is controlled by reduced daylength in the autumn, but also to species in which extension growth is not normally photoperiodically controlled. It has been pointed out that the seedlings of certain species ultimately cease extension growth even when maintained under constant long-day conditions, apparently because growth becomes limited by some endogenous mechanism. Nevertheless, in these species, the total amount of extension growth attained is affected by the daylength conditions under which the plants are maintained, and under various constant photoperiods the duration of growth increases progressively with the length of the photoperiod. In such species, although the seasonal growth attained in nature will be controlled primarily by an endogenous mechanism

(determined by the genotype) it will also be affected by the total nexus of factors constituting the external environment, including the daylength conditions. Presumably, selection operates upon the genotype to give the optimum duration of extension growth under the daylength conditions prevailing in any given region. If, however, such a species is transferred to a new region of different latitude, the mean daylength conditions throughout the period of growth will be altered and will affect the duration of extension growth. Thus, any species showing photoperiodic responses with respect to extension growth will be affected by changes in latitude, even if extension growth in a given species is not normally controlled by seasonal changes in daylength. In introducing exotic species, it is therefore necessary to select genotypes having a duration of extension growth appropriate to the frost free period, when grown under the daylength conditions of the new region.

Several clear instances of natural adaptation to daylength conditions are known for woody species. Thus, whereas species of *Salix*, *Betula*, *Ribes*, *Vaccinium*, etc. from lower latitudes cannot form winter resting buds under continuous illumination, Schulz (68) found that the corresponding subarctic species of these genera are able to do so. Similarly, several woody species, whose distributions cover a wide range of latitude (and hence of daylength conditions), have been shown to exhibit ecotypic variation with respect to photoperiodic response.

The existence of such ecotypic variation was first clearly demonstrated by Sylven (70) for *Populus tremula*. He found that when races from northern Sweden were grown in a more southern station they showed reduced growth and stunting compared to races indigenous to that locality, while southern races grown in the far north showed delayed dormancy and were subject to damage by autumn frosts. He showed that these genetically controlled differences in response of the various ecotypes are essentially of a photoperiodic nature.

Similar ecotypic variation within the North American species *Populus tacamahaca*, *P. deltoides*, and *P. trichocarpa* has been demonstrated by Hoffman (31) and Pauley & Perry (59), who have shown that the time of cessation of extension growth was inversely correlated with the latitude of origin. Photoperiodic ecotypes have also been reported by Vaartaja (73) to occur in *P. silvestris* and *Alnus incana*.

Ecotypic variation has been reported within species of *Populus* collected from the same latitude, but from different altitudes (59). Although these ecotypic differences do affect the photoperiodic responses of the various races, they are probably to be regarded as primarily adaptations in the endogenous mechanism determining the duration of extension growth with respect to the length of the frost free period and other external conditions. They are probably not essentially adaptations in photoperiodic response, although such genetic differentiation will, of course, affect the photoperiodic responses of the various races as observed under experimental conditions. Similar considerations would seem to apply to the altitudinal races of *P.*

silvestris (35, 86), although in this species the processes affected are internode extension, leaf growth, etc., rather than the duration of extension growth. The photoperiodic ecotypes of woody species have sometimes been distinguished as "long-day" or "short-day" types, but such terms cannot be used in the same sense as for the flowering response of herbaceous species. In the latter, changes in the daylength conditions have opposite effects in long-day and short-day plants, whereas in different photoperiodic ecotypes the responses change in the same direction and differ only quantitatively.

Although in many cases the frost resistance of a given species can be related to the duration of extension growth, nevertheless, instances were observed by Moshkov (53), in which photoperiodic conditions affected frost resistance independent of any effect upon the duration of extension growth. Thus, it was found that maximum frost resistance occurred in seedlings of *Juglans regia* and *Prunus armeniaca* when grown under photoperiods of 14 to 15 hr., whereas seedlings grown under longer or shorter daylengths were highly susceptible to frost damage, although the duration of extension growth was essentially the same under all photoperiods. Thus, it would seem that although the photoperiodic conditions may produce no visible responses in a given species, nevertheless they may still have an effect on its frost resistance. One way in which this may occur is through an effect on the dormancy of the cambium, since a nondormant cambium is liable to damage by autumn frosts. The possibility cannot be excluded, however, that daylength conditions not only bring about dormancy of meristematic tissue, but may also control various processes affecting frost resistance in differentiated tissue. Thus, Mollart (51) observed that the storage of starch in the wood parenchyma of *R. pseudacacia* is much greater under short-day than long-day conditions. Photoperiodism may, therefore, still be of great biological significance, even in those species in which the visible growth processes are not photoperiodically controlled.

It is clear that photoperiodism has important implications for the breeding of forest trees, for new types must show the same delicate adjustment to daylength as is found in natural ecotypes. Moreover, by appropriate photoperiodic treatment it may be possible to reduce the time delays involved in tree breeding, by shortening the juvenile period. Doorenbos (21) has already demonstrated the value of photoperiodic breaking of dormancy for hastening the breeding of *Rhododendron*.

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PLANT PROTEINS[1]

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During the last decade many papers on plant proteins have been published. One of the reasons has been the development of new and powerful methods for analyzing high-molecular-weight substances, especially ultracentrifugation (1) and electrophoresis (2). Old problems in the field of plant proteins, which were impossible to solve with earlier methods of investigation, are now treated with the new ones. As a result of this new interest in the plant proteins, both the old and the more recent experimental findings on this subject have been reviewed from different point of view. Chibnall (3) in his review on the nitrogen metabolism in plants, drew special attention to the problems in this field. The reviews by McCalla (4, 5), Bonner (6) and Lugg (7) should also be mentioned. During the last few years some very comprehensive reviews have been published, especially one by Steward & Thompson (8) on the protein metabolism in the plant. Recent knowledge of the leaf proteins was reviewed in an earlier volume of this journal by Wildman & Jagendorf (9). The seed proteins, finally, were reviewed by Brohult & Sandegren (10).

Under these circumstances for this review to cover the whole subject of plant proteins would seem to be an unnecessary duplication. Instead, I have chosen to treat some special problems of importance to our knowledge of the plant proteins and to examine critically a few experimental studies which have given diverging results.

THE CLASSIFICATION OF THE PLANT PROTEINS

The plant proteins have been classified in many different ways. One method, based on the occurrence of the proteins, classifies them as leaf proteins, seed proteins, proteins in stems, roots, etc. This method is often useful but sometimes is not sufficiently clear. Another method, used by Steward & Thompson (8), distinguishes between metabolically active and inactive proteins. Accordingly the seed proteins are referred to as metabolically inactive or reserve proteins. Seeds contain in addition, however, enzymes and nucleoproteins, which are metabolically active during the ripening and germinating processes but are metabolically inactive during the dormant period. This change may cause some confusion.

Osborne's (11) classification, based on solubility, is still one of the more useful methods, even if the borders sometimes are not clearly defined as in his characterization of the globulins. According to Osborne, globulins are insoluble in water, but soluble in dilute salt solutions. Several "globulins" from different plant sources, however, require salt solutions of different ionic strengths to be completely soluble.

[1] The survey of literature pertaining to this review was concluded in May, 1955

Another method of classification, although unsuitable for plant proteins, but good for serum proteins, is based on the fact that globulins are salted out by $(NH_4)_2SO_4$ at 50 per cent saturation and the albumins are precipitated at 100 per cent saturation. Several investigations on seed globulins (Osborne's definition) have shown that many globulins are precipitated first at 60 to 70 per cent saturation with $(NH_4)_2\ SO_4$ (12, 13). Thus, the last two methods of classification are insufficiently precise.

Proteins are also classified as simple and conjugated (e.g., with active groups) and in many other ways. The different methods of classification have caused confusion. Divergent results in some experimental work are based on a misunderstanding of definitions. In the present paper Osborne's definitions of albumins and globulins will be used.

THE HOMOGENEITY OF SEED PROTEINS

One fundamental problem in the chemistry of plant proteins is whether or not these proteins consist of individual, well defined units. Throughout the time these substances have been studied the discussion on this point has been animated. Most investigations on the homogeneity of the plant proteins have been carried out on seed proteins because seeds are generally rich in easily extractable proteins. Furthermore, seeds are very interesting biologically as pointed out by Vickery (14): "the single plant seed, the ultimate source of all foodstuffs, is one of the most important organisms with which mankind is concerned." Seed proteins serve mainly as reserve or storage proteins which are digested enzymatically during the germination process. The amino acids and peptides formed are transported to the growing parts of the new plant where they become available for protein synthesis (3). Some of the seed proteins, however, serve other purposes, e.g., as enzymes which assist in the breakdown or synthesis of proteins, starch, and other products.

Osborne's Work on Seed Proteins

The seed proteins have been studied for more than 200 years beginning with Beccari's investigation of gluten from wheat in 1747. However, we now know that the methods used for the preparation of seed proteins during the nineteenth century denatured the proteins. The fundamental work in the field of seed proteins was done by Osborne, who started in 1891 and during the following 30 years published more than a hundred papers on this subject [for references see (11)]. He and his co-workers used milder methods of preparation so that many of their results are still valid. In his earlier experiments he worked on the hypothesis that closely related plants contained identical seed proteins (14). Later, in collaboration with Wells, he was able to show by immunological tests that there are few, if any, seed proteins which are identical (15 to 18).

It is, however, very hazardous to make valid conclusions from immunological tests on seed proteins. The proteins must be completely free from foreign peptides, carbohydrates, etc., which can give immunological reactions. Osborne's results, however, are probably correct, because it seems

very improbable that different plants can synthesize proteins with exactly the same amino acid sequence. If only the rough physical and chemical properties are considered, e.g., molecular weight, electrophoretic behaviour, amino acid content, etc., the truth is between the first and the last working hypothesis of Osborne. It is, however, impossible to draw any definite conclusion from Osborne's immunological experiments, because no criterion of the purity of their protein fractions is available.

Osborne showed that the seed globulins from different plants could be separated into a few simple fractions, each probably containing only one globulin. Osborne thought that the different globulins were homogeneous and that they consisted of individual proteins. From seeds of *Pisum sativum*, *Ervum lens*, and *Vicia faba* he isolated a globulin component which he called legumin. The elementary composition (C, H, N, S) of the legumin from all three plants was identical. The same was true for another globulin component, called vicilin. But the composition of legumin was different from that of vicilin. Legumin contained 0.40 per cent S while vicilin contained only 0.16 per cent S (19).

These results published 60 years ago by Osborne *et al.*, indicated that the seed globulins could be separated into a few simple fractions; however, Osborne used extraordinarily crude methods, based on solubility and elementary chemical composition, for the analysis of purity and homogeneity of his protein fractions. Thus, when new methods of protein analysis were developed, it was natural that Osborne's fascinating hypotheses were re-investigated. The recent experimental work in this field has not confirmed all of Osborne's results. This is because the separation of the proteins has not been carried out in the proper way. To prove this statement, some examples will be given in the present paper.

Proteins in Monocotyledonous Seeds

Because the cereals are very important in nutrition the proteins in the seeds of different species of the Gramineae have been investigated repeatedly. Most of the work has been on wheat seeds because they contain a special protein fraction, gluten, which is of fundamental importance in the baking properties of wheat flour. The results of the investigations on gluten are not treated in detail in the present paper, since this has been done by McCalla (5). Osborne (11) classified the wheat proteins into four main groups: glutelin, gliadin, globulin, and leucosin. The main fraction consists of the polydisperse gluten. A definite molecular weight for gluten cannot be determined because the protein molecules are dissociating and associating (20). Rich (21) suggests that the whole wheat protein should be regarded as a single substance which can be split into arbitrary fractions. This suggestion is contradicted by some experiments on the globulins in barley and wheat. Although the globulins make up only 6 to 10 per cent of the total wheat protein they are of fundamental importance for the seed and thus definitely worth special investigation.

Quensel (12) investigated the seed globulins of barley, *Hordeum vulgare*,

in the ultracentrifuge using Osborne's preparative methods, i.e., extraction of the seed material with salt solutions at pH 7 followed by precipitation of the globulins with $(NH_4)_2SO_4$ at different degrees of saturation. The precipitates were dissolved in salt solutions and then repeatedly dialysed against water to the salt-free state. In this way Quensel isolated four globulin components, called α,β,γ, and δ from seeds of barley. Only the α and γ components could be completely separated from low-molecular-weight material, as shown by ultracentrifugation. The α and γ components could never be completely separated with the methods used by Quensel, e.g., differential centrifugation in an air-driven centrifuge. The relative concentration of the two components could be changed, however, Thus, Quensel's results on the barley seed globulins demonstrated the existence of several distinct components, but it did not provide any answer to the fundamental questions whether or not these components belong to a dissociating system and whether these globulins are differentiated in the seeds before the extraction. The problem of the individuality of the globulins in the cereals was solved in a study on the wheat proteins.

The globulins in wheat seeds are more soluble than those in barley, and the solutions of the wheat globulins are practically colourless. With barley, the protein solutions are dark brown in colour which complicates optical observations in ultracentrifugation, electrophoresis, and diffusion experiments. The primary reason for choosing seeds of wheat for these experiments was, however, an observation by Osborne (11), which indicated that different fractions of the wheat seed could be studied:

> Extracts of seeds always contain in addition to a relatively large amount of one or two types of protein, which are manifestly the reserve protein of the seed, a certain small proportion having distinctly different properties. It is probable that most of this latter protein is yielded by the cells of the embryo as well as by the protoplasm of the endosperm cells. With most seeds definite evidence of this has not yet been obtained, but in the case of wheat the embryos are separated by the commercial process of milling in a nearly pure condition, and a study of this product has shown that those proteins which are obtained only in small quantity from the entire seed are present in relatively large amount in this embryo meal.

The globulins of wheat were studied along these lines by Säverborn *et al.* (22). They found that wheat seeds contained only two globulin components with the same sedimentation constants as the α and γ components of barley. The γ component was isolated in a pure state from wheat embryo. The molecular weight was 213,000. The relative concentrations of the two components changed when the extraction was carried out on the endosperm (flour) fraction compared to the bran fraction. The sedimentation diagrams are shown in Figure 1.

The γ component from wheat embryo has been isolated in large amounts (about 10 gm. in each preparation), and the purified globulin has been dried in the frozen state. The dried preparation can be redissolved in salt solutions and gives exactly the same sedimentation diagram as before drying. Under

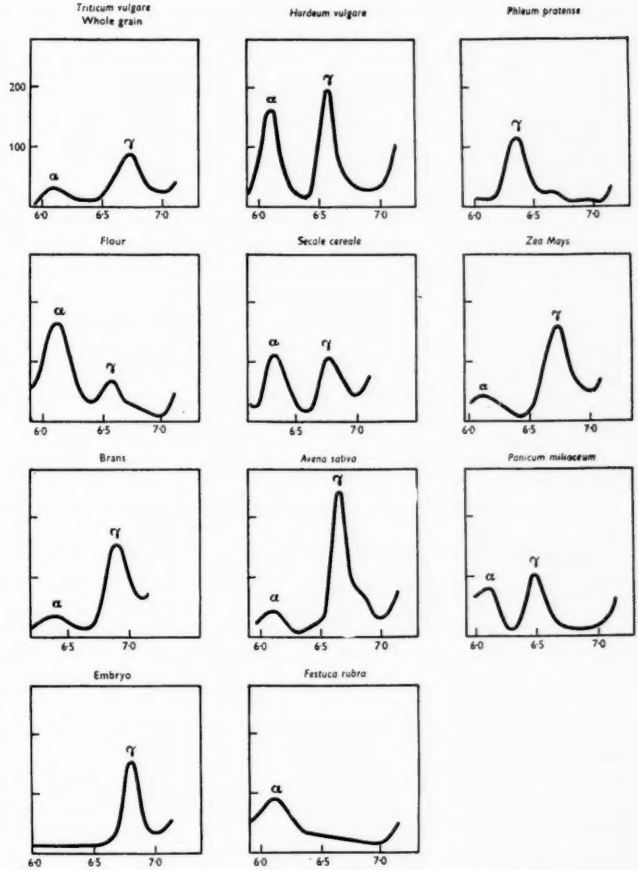

FIG. 1. Sedimentation diagrams showing the composition of globulin solutions prepared by extraction of seeds from different species of the Gramineae. Note, however, that the four diagrams to the left refer to different fractions of seeds from the single species, *Triticum vulgare*.

the experimental conditions used, i.e., neutral solutions and an ionic strength of 0.3, only one component was obtained in the ultracentrifuge.

In later experiments, Danielson (13) purified the α component from the endosperm of barley seeds. A determination of the molecular weight gave the value of 29,000.

The preceding results clearly show that globulin components can be

isolated from seeds of barley and wheat which are homogeneous in the ultracentrifuge. An important fact is that these globulins, which are impossible to separate with conventional methods, are already differentiated in the seed. It is very probable that the different globulin components serve different purposes in the seeds during the germination process, since the embryo is very rich in these well-defined globulins.

The globulins in the seeds of wheat and barley make up only a small fraction of the total proteins. Nevertheless, it is very important from a theoretical point of view that it is possible to isolate homogeneous protein components from seeds of different species of the Gramineae. These experiments show that Rich's hypothesis (21) that the whole wheat protein consists of a single substance must be modified in some way.

Much more work must be carried out on globulins in the seeds of barley and wheat to get answers to many important questions. A protein cannot be characterized as homogeneous only on the basis of data obtained in the ultracentrifuge. Thus, a complementary electrophoretic investigation should be carried out. A quantitative study of the amino acid composition of the α and γ globulins from barley and wheat may give us more knowledge about the homogeneity and individuality of these proteins. Jensen (23) determined the amino acid composition of the β component from barley seeds, but his experiments were not extended to the other components.

Whether or not other species of the Gramineae contain the same globulin components as those occurring in barley and wheat was studied by Danielson (13). The sedimentation diagrams are shown in Figure 1. In seeds of *Secale cereale*, *Avena sativa*, *Zea mays*, and *Panicum miliaceum*, globulins corresponding to the α and γ components could be isolated. Seeds of *Festuca rubra* contained the α component only. The γ component could be isolated from seeds of *Festuca pratensis* and *Phleum pratense*. These investigations are, however, too schematic to give definite support to Osborne's hypothesis that closely related plants contain similar seed proteins. Careful determinations of the amino acid composition of the globulin fractions from seeds of different species must be carried out before this important question can be answered in a definite way. The experimental work is rather difficult, since the globulins are present in low concentrations in the seeds.

The Proteins in Dicotyledonous Seeds

McCalla (5) states in his review on plant proteins: "Most plant seeds contain more than one protein but these appear to belong to the albumins and globulins and are probably much more distinct and clearly defined than are the so-called prolamines and glutelins of cereals, although not so clearly defined as the older work would lead us to believe." From what has so far been found in the recent studies on the seed proteins from species not belonging to the Gramineae with modern physical-chemical methods of analysis, very few observations have been made which are opposed to McCallas' characterization. The opinion that the proteins in seeds from different species

are more complicated than was earlier believed can, however, lead to incorrect conclusions based on insufficient experimental material, which will be shown in the following example from investigations on the pea proteins.

Pea proteins.—The seeds of *Pisum sativum* are very rich in protein. Osborne and his co-workers (24, 25) isolated three protein fractions which they thought to be homogeneous, well-defined proteins. Two globulins, which they called vicilin and legumin, and one albumin called legumelin, were found. The globulins were characterized as follows: (*a*) legumin does not coagulate and is not precipitated when a solution of this protein is heated to 100°C.; (*b*) vicilin coagulates upon heating to 95 to 100°C. and is soluble in more dilute salt solutions than legumin. The globulins were isolated in the following way. The proteins were extracted from the ground seed material with 10 per cent NaCl solution and precipitated from the extract by saturation with solid $(NH_4)_2SO_4$. The precipitate was dialyzed against water so that the proteins dissolved as the ionic strength was lowered. When the salt concentration upon prolonged dialysis reached a lower limit, the globulins precipitated. Legumelin was isolated from the supernatant solution of the dialysis against water. In the first experiments (24) the globulin precipitate was dissolved in NaCl solution, which was diluted in several stages. In this way legumin precipitated at higher ionic strength than vicilin. In later experiments (25) precipitation with $(NH_4)_2SO_4$ to 60 per cent saturation was used. According to Osborne & Harris, legumin is precipitated by this method, but vicilin is not.

Osborne's methods of separation were checked simultaneously on the pea proteins in two different places. In both, modern methods of protein analysis were used. Danielson (13) used ultracentrifugation and Wetter & McCalla (26) employed electrophoresis. The two experimental studies gave highly divergent results, which therefore need explanation.

The study of the pea proteins in the ultracentrifuge showed two globulin components (13). Separation experiments according to Osborne & Harris (25) gave no complete separation, but one globulin component, legumin, could be reasonably purified by precipitation with $(NH_4)_2SO_4$ at 60 per cent saturation. Dilution with water according to Osborne & Campbell (24) all gave unsatisfactory results, but vicilin could be partly purified by this procedure. These preliminary experiments showed, however, that Osborne and his coworkers had, in some cases, been working with globulin preparations where the separation of legumin and vicilin had been well along. It seems improbable however, that they had obtained a complete separation of the two globulins. From the results obtained by ultracentrifugation, it is clear that the differences in chemical composition between legumin and vicilin observed by Osborne & Campbell (19) are based upon analyses carried out on protein preparations which were not completely homogeneous. If legumin and vicilin are completely separated, the difference in sulphur content must be more pronounced than was found by Osborne & Campbell.

The two globulins were completely separated by isoelectric precipitation

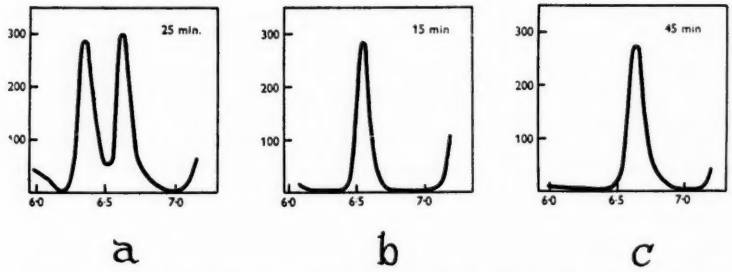

FIG. 2. Sedimentation diagrams after separation of vicilin and legumin by isoelectric precipitation. Left (a): initial solution; centre (b): precipitate at pH 4.5 (legumin); right (c): supernatant at pH 4.5.

of legumin at pH 4.5 where vicilin is soluble and legumin is not (13). Ultracentrifuge diagrams showing the purified globulins can be seen in Figure 2. The molecular weights were determined to be 331,000 for legumin and 186,000 for vicilin. The isolated globulins are also homogeneous when investigated with electrophoresis (27), which can be seen from Figures 3 and 4. The isoelectric points were found to be pH 4.8 for legumin and pH 5.5 for vicilin. The results of the electrophoretic study have recently been confirmed by Reznichenko (28), who showed that the pea globulins consist of two electrophoretically homogeneous components, and by Kretovich and co-workers (29), who separated legumin and vicilin. The isolated components were shown by Kretovich *et al.* to be electrophoretically homogeneous. The reproduction of the electrophoretic diagram for vicilin at pH 9.0 [(29) Figure 2)] fulfils great demands upon electrophoretic homogeneity. Kretovich *et al.* also investigated the albumin fraction, called legumelin by Os-

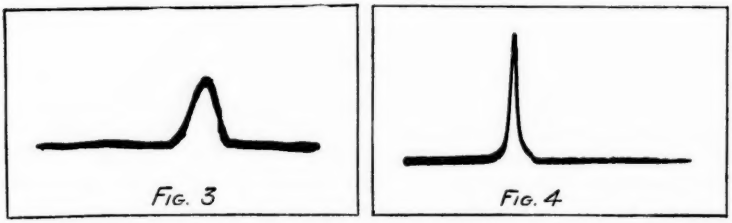

FIG. 3. Electrophoretic diagram of vicilin. pH 4.33. Concentration 0.7 per cent. Potential 3.01 volts/cm. Migration at +5°C. for 380 min. to the right. The descending peak has migrated 2.0 cm. in the apparatus. $\theta = 70°$. θ is the angle of inclination of the diagonal slit; this equals zero in the vertical position.

FIG. 4. Electrophoretic diagram of legumin. pH 3.70. Concentration 1.7 per cent. Potential 0.99 volts/cm. Migration at +15°C. for 395 min. to the right. The descending peak has migrated 1.4 cm. in the apparatus. $\theta = 40°$.

borne. Four electrophoretically distinct components were found in the albumin fraction. It is, however, very probable that two of these components consist of legumin and vicilin.

In their investigation on pea proteins, Wetter & McCalla (26) always obtained four components when the different fractions were analyzed by electrophoresis. They did not succeed in separating these components, a fact which seems remarkable considering what has been said above. There are, however, several reasons why Wetter & McCalla failed in their separation experiments. In the first place, they extracted the ground seed material for 48 hr with ether in a Soxhlet apparatus. This extraction is not necessary for the separation of the globulin components, and it is not possible to say what effect boiling organic solvents have on the well-defined proteins. Secondly, all the separation experiments were carried out directly on the extracts with no reprecipitations. It is almost impossible to separate the globulins from the albumins this way. The experience of the present author is that a preliminary separation of all the proteins from all substances of nonprotein nature is very important in all separation experiments. Amino acids, peptides, and carbohydrates, for example, can act as protective colloids and make the desired separations more difficult. In one experiment [(26) Figure 2 and Table II], Wetter & McCalla showed that there are possibilities for separation of the components. They carried out precipitations with $(NH_4)_2SO_4$ at 60 per cent saturation at different pH values, 6.75, 5.50, 5.20, and 4.20. At pH 6.20 only a small amount of one component, called d, is obtained. By lowering the pH, the concentration of this component increases as can be seen from the electrophoresis diagrams. Thus it seems probable that this component consists of legumin. A comparison of the electrophoretic mobility of this component with the values obtained by Danielson (27) demonstrates the validity of this assumption. For this comparison a correction factor must be used, because the experiments were carried out at different temperatures. The comparison with Danielson's results also shows that the component called b by Wetter & McCalla consists of vicilin.

The studies on the pea proteins described above clearly show the importance of using the most suitable methods of preparation when doing separation experiments on plant proteins. Thus, it may be dangerous to state that a mixture of proteins is impossible to separate unless the statement is based on a very large amount of experimental material. In the present author's opinion, McCalla has gone too far when he says in his review (5) on the basis of Wetter & McCalla's electrophoretic studies on pea proteins: "Thus pea proteins, like most others studied, are mixtures of components, but these cannot be satisfactorily separated by usual methods of separation." By using careful methods of separation, the reviewer, as well as Kretovich and co-workers, have shown the separation of the pea globulins to be possible, and the results must be seen as a triumph for the skillful work of Osborne nearly 60 years ago. It cannot be denied however, that analysis of proteins by ultracentrifugation and electrophoresis still are rather crude

methods of analysis. In the near future we will probably have more powerful tools for the study of the homogeneity of proteins, and these methods will perhaps show that the pea proteins which are homogeneous both on ultracentrifugation and electrophoresis may consist of complex mixtures.

One aspect of this problem has been discussed by Kretovich *et al.* in their paper on pea proteins (29). They found that the globulin fractions isolated from pea seeds were enzymatically active. The vicilin fraction had catalase, peroxidase, and dipeptidase activity, and legumin had catalase, invertase, and dipeptidase activity. The albumin fraction also had carboxylase activity. When the same authors isolated these protein fractions using Osborne's methods of preparation, no enzymatic activity could be observed with the exception of the albumin fraction which showed a weak catalase activity. From these findings Kretovich *et al.* draw some very pretentious conclusions. They state that pea seeds do not contain any pure reserve proteins, i.e. the seeds have no metabolically inactive proteins. Kretovich *et al.* found vicilin and legumin to be enzymatically active and thus these proteins are metabolically active. It is difficult to accept this hypothesis before more experimental work has been carried out.

Kretovich *et al.* stress that their method of preparation is superior to that of Osborne because it does not denature the proteins, and that therefore, they can isolate globulins with enzyme activity. The present author believes, however, that the albumin fraction contains all the enzymes of the seeds, and that the globulins are pure reserve proteins. If the globulin fractions contain small amounts of albumins, invisible in the electrophoresis diagrams, these contaminations may give a measurable enzymatic activity, especially in the case of catalase which has a very high turn-over number. However, this hypothesis does not explain why the albumin fraction isolated by Kretovich *et al.* was enzymatically inactive. It is not impossible that an inactivation occurred during the drying of the purified legumelin fraction. The enzyme activity may be more stable when the albumins are dried in the presence of large amounts of globulins.

A critical examination of the separation methods used by Kretovich *et al.*, shows clearly that the globulins may be contaminated with albumins. The pea meal is extracted with 0.2 per cent NaCl at pH 7.0, and the clarified extract is directly dialyzed against water which precipitates the globulins, vicilin and legumin. Vicilin is dissolved according to Danielson (30) by treating the globulin precipitate with 0.2 *M* NaCl at pH 4.7, and is again precipitated by dialysis against water and dried in the frozen state. Vicilin prepared according to this method may be contaminated with albumins. The same is true for legumin as prepared by Kretovich *et al.* Danielson (30) first removed most of the albumins by precipitation of the globulins with $(NH_4)_2SO_4$ at 70 per cent saturation. The globulins were then completely separated from the albumins by two successive dialyses against water. The globulins isolated by this method had no enzyme activity. From what has been said here it is quite clear that a close study of this problem must be

carried out before making any conclusions of fundamental importance.

From a botanical point of view, Osborne's hypothesis that closely related plants contain similar seed proteins is a fascinating one. To get some ideas about this problem the seed globulins of several different species of the Leguminosae, chosen at random, were investigated in the ultracentrifuge (13). Practically all the species contained two globulin components with sedimentation constants of the same order of magnitude as for vicilin

TABLE I

SEDIMENTATION CONSTANTS (s_{20}) OF THE GLOBULIN COMPONENTS OF VARIOUS LEGUMINOSAE*

Acacia alata	1.3	7.9	11.6	—
A. decipiens	—	8.0	12.7	—
A. farnesiana	—	8.0	—	—
A. longifolia	2.7	7.6	—	18.0
A. penninervis	1.5	7.4	—	18.8
A. saligna	—	7.8	13.7	—
A. verticillata	—	7.8	—	—
Arachis hypogaea	2.0	8.4	13.1	—
Astragalus galegiformis	—	8.3	13.2	—
Cytisus laburnum	—	8.1	14.0	—
C. supinus	1.8	8.0	13.4	—
Dolichos lablab	—	7.3	11.7	—
Ervum lens	—	7.3	13.2	—
Genista tinctoria	—	8.5	13.3	20.3
Glycine soja	—	8.0	13.1	—
Lathyrus clymenum	—	7.6	13.0	—
L. odoratus	—	7.6	12.0	—
L. sativus	—	7.5	13.0	—
L. silvestris	—	7.5	13.0	—
Lotus tetragonolobus	—	8.3	13.1	—
Lupinus albus	—	8.2	12.3	—
L. angustifolius	—	8.2	13.1	—
L. luteus	—	8.3	11.5	—
L. polyphyllus	—	8.7	12.2	—
Medicago sativa	—	6.8	11.4	—
Phaseolus coccineus	4.3	7.4	12.2	—
P. nanus	—	6.6	10.1	—
P. vulgaris	4.9	7.3	11.0	—
Pisum sativum	—	8.1	12.6	—
Trifolium hybridum	—	7.7	12.9	—
T. pratense	—	7.7	11.2	—
T. repens	—	7.3	—	18.2
Vicia faba	—	7.1	11.8	—
V. sativa	—	7.1	11.9	—

* Results in Svedberg units.

($s_{20}=8.1$ S) and for legumin ($s_{20}=12.6$ S) as can be seen from Table I. Some proteins with lower ($s_{20}\cong 2.5$ S) and higher ($s_{10}\cong 18.5$ S) sedimentation constants were observed. By heating a solution of legumin from peas, a component with $s_{20}\cong 18.5$ S is easily obtained. Thus, it is very probable that in those cases where a component with $s_{20}\cong 18.5$ S was observed, this component was an association product from the component with $s_{20}=12.6$ S.

Thus, if only particle size is considered, all the species investigated contained seed globulins similar to those in *Pisum sativum*. Before drawing any conclusions concerning more subtle similarities between the proteins from different species much more preparative work must be carried out. In the first place, separation experiments must be performed on the globulins from different species, using the same methods as were worked out for the pea globulins. When a complete separation has been obtained, a complete quantitative amino acid analysis according to Moore & Stein (31) should be carried out on the isolated globulin components. The homogeneity should also be studied electrophoretically.

Proteins in lupin seeds.—Recently an interesting study of the proteins in seeds of *Lupinus angustifolius* was published by Joubert (32). As can be seen from Table I, Danielson (13) observed two globulin components with $s_{20}=8.20$ S and $s_{20}=13.05$ S, but he did not try to separate the two components. Joubert mainly used the separation method which was developed for the pea proteins, and he obtained a fairly good separation of the two globulins. In no case, however, was a complete separation obtained, because Joubert did not use successive isoelectric precipitations as described for the pea proteins (30). The preliminary separations obtained by Joubert clearly show that a complete separation is possible. He analyzed the proteins by ultracentrifugation and electrophoresis. The molecular weights were determined to be 181,000 and 336,000, i.e., practically the same values as were obtained for the pea globulins. Joubert points out the similarities between the proteins in peas and legumin seeds:

> The properties of the two globulins of lupin seeds are very similar to that of vicilin and legumin from seeds of peas, reported by Danielson. Not only are the molecular weights very similar, but the method of fractionation applied to the separation of the pea globulins gave almost as good results when used for the fractionation of lupin seed globulins.

Joubert says in his paper that a large research program has been started on the proteins in lupin seeds. It is to be hoped that purely chemical determinations will be carried out on the isolated globulins. Danielson & Lis (33) observed different contents of tryptophan, arginine, histidine, lysine, glutamic acid, and aspartic acid in vicilin and legumin from peas. It would be very interesting to know whether the same differences could be observed in the globulins from lupin seeds, and whether there are any differences in chemical composition between the globulins from the two plant species.

SEED GLOBULINS AS DISSOCIATING SYSTEMS

Peanut globulins.—Joubert's results with lupin seed globulins give new interest to the question of seed globulins as dissociating and associating systems. Much experimental work has been carried out on this problem. The most fundamental results have been obtained by Johnson and coworkers (39–42). They state, proteins in seeds of peanuts, *Arachis hypogaea*, consist of a mixture of components which associate and dissociate under some experimental conditions. Without a doubt this assumption is correct, and, as far as the reviewer knows, their results have never been criticized. During the last few years, however, new knowledge has been obtained in this field of protein chemistry which justifies a reconsideration of the whole problem of proteins as complex mixtures. Johns & Jones (34, 35) found that the peanut globulins could be separated into two fractions, called arachin and conarachin, which differ in their content of suphur, basic nitrogen, and amino acids. Jones & Horn (36) described two preparation methods for arachin, which is the main fraction. Irving *et al.* (37, 38) showed by electrophoresis that these preparation methods give two different products. Johnson (39) demonstrated by ultracentrifugation that a practically homogeneous globulin is obtained using one of Jones's and Horn's methods for isolating arachin. The globulins were extracted with a salt solution and arachin precipitated with $(NH_4)_2SO_4$ at 40 per cent saturation. The sedimentation constant of the homogeneous arachin fraction is $s_{20}=14{,}6$ S, but, if a solution of this protein is diluted with water and at the same time acidified to pH 5.0, arachin is dissociated into molecules with $s_{20}=9.5$ S. Johnson *et al.* have carefully studied this dissociation process both in the ultracentrifuge and with electrophoresis (40, 41). They extract the defatted peanut meal with 6 per cent NaCl. The extract gives only one component with $s_{20}=14.4$ S upon ultracentrifugation (40). The authors conclude that arachin is present in this form in the native state in the seeds. The extract is precipitated with $(NH_4)_2SO_4$ and the precipitate is dissolved in phosphate buffer of ionic strength 0.1 and pH 7.98. By this method the arachin component with $s_{20}=14.6$ S is obtained in a pure state. In order to dissociate this component they dialyse the solution against distilled water. The pH is maintained at 5.0 by adding HCl to the water. The resulting precipitate is dissolved in phosphate buffer, pH 7.62, and gives two components in the ultracentrifuge. The sedimentation constant of the main component is $s_{20}=8.3$ S and of the other component $s_{20}=13.4$ S. Corresponding results are obtained in electrophoresis experiments.

Johnson & Naismith (42) have also studied the conarachin fraction from peanuts. It consisted of several components, and it was impossible with the methods used to isolate any of these components in a pure state. The main component, with $s_{20}=8.7$ S, is precipitated with $(NH_4)_2SO_4$ between 65 and 85 per cent saturation. Reprecipitation gives a main component of 80 per cent purity. It is always contaminated with low-molecular weight material

with $s_{20} \cong 2$ S, resembling the albumin fraction from peas. The impossibility of separating the components in the conarachin fraction shows that there is some fundamental difference between the peanut proteins and the pea protains. Johnson & Naismith observed an association reaction when a solution of conarachin was diluted. This association took place only at ionic strengths below 0.35.

Thus, in order to get dissociation in the case of arachin or association in the case of conarachin, Johnson and co-workers have worked at very low ionic strengths. All ultracentrifugations and electrophoresis experiments have been carried out at ionic strengths of 0.04 to 0.1. At these low salt concentrations arachin has a low solubility, and it is only possible to use a narrow pH range of 7.0 to 9.0. This way of working is not the common one when dealing with plant proteins. Here the important question of how globulins should be defined must be answered. It is impossible to have any definite border at a particular ionic strength when using Osborne's definition of globulin as a protein which is soluble in dilute salt solutions but insoluble in water.

Dissociating lupin globulins.—In his study of the lupin globulins, Joubert (32) made some interesting observations at low salt concentrations. A solution containing the component with $s_{20} = 11.6$ S and corresponding to legumin from peas was dissolved in a buffer at pH 7.0 with an ionic strength of 0.31. This solution was dialyzed overnight against a buffer at pH 8.8 with an ionic strength of 0.1. By this treatment the main part of the protein was dissociated into smaller molecules with $s_{20} = 7.1$ S. The dissociated molecules were not identical, however, with the component with $s_{20} = 7.8$ S, which corresponds to vicilin in peas. The dissociated molecules are completely associated if the solution is dialyzed overnight against a buffer at pH 7.0 with an ionic strength of 0.31. Exactly the original sedimentation diagram is obtained. A corresponding experiment with vicilin from lupin seeds did not give any dissociation or association reactions. Joubert's experiments at both high and low ionic strengths on the same protein solution are very important for our understanding of the homogeneity of the seed globulins. He has clearly shown that a globulin component which is homogeneous from a physical-chemical point of view at high ionic strength can be polydisperse at low salt concentrations and that this reaction is reversible. One aspect of this problem is the question of the native state of the globulins in the seeds. Johnson's conclusion (40) that arachin occurs in the associated state in the seeds because it is extracted from the seeds in that state with 6 per cent NaCl is not a realistic one. First, the actual ionic strength in the cells must be determined. Second, it is not very probable that the globulins occur in a soluble state in the seeds (e.g., in the aleuron layer of some cereals the proteins occur as crystals).

From a theoretical point of view it is interesting that the seed globulins with molecular weights in the order of 300,000 to 400,000 are easily broken down to molecules of about half of their original size. One of the goals of the protein chemists is to explain the mechanism of this process.

Soya bean proteins.—Some recent observations, from a physical-chemical point of view, carried out by Naismith (43) on the proteins in soya beans, are of interest in the discussion of seed proteins as dissociating systems. From seeds of soya bean Osborne & Campbell (44) isolated a protein fraction, called glycinin, by dialysis of a 10 per cent NaCl extract. The precipitate consists of glycinin. By fractional precipitation with $(NH_4)_2SO_4$ Jones & Csonka (45) showed that the properties of the globulins varied with the methods of preparation. Briggs & Mann (46) studied the glycinin fractions prepared according to Jones & Csonka electrophoretically. The glycinin was shown to be heterogeneous on electrophoresis. Hartman & Cheng (47) prepared glycinin by isoelectric precipitation at pH 4.5.

Naismith checked the purity of glycinin, prepared according to the methods described above, in the ultracentrifuge. In no case were monodisperse glycinin preparations obtained. A 10 per cent NaCl extract, dialyzed against a buffer at pH 7.8 with an ionic strength of 0.5, gives by ultracentrifugation three components with $s_{20}=6.6$, 10.6 and 15.0 S. Naismith partly purified the main components by fractional precipitation with $(NH_4)_2SO_4$. The heavier component, $s_{20}=10.8$ S, precipitates between 65 and 70 per cent saturation and the other component, $s_{20}=6.7$ S, is obtained in high concentration by repeated precipitation of the fraction which precipitates between 70 and 85 per cent saturation. The components are not obtained completely pure, but a complete separation seems to be possible. It is surprising that Naismith did not try other methods of separation on the partly purified fractions, e.g., isoelectric precipitation of the component with $s_{10}=11$ S according to Hartman & Cheng (47). Further, the method of Briggs & Mann (46) could have been used on the partially purified fractions. By this method the component with $s_{20}=11$ S is precipitated by cooling a solution of the protein mixture.

Naismith also carried out experiments at low salt concentrations. Both globulins are associated into larger molecules at low ionic strengths. In this respect, the soya bean globulins are similar to conarachin from peanut seeds, but differ from the arachin fraction which dissociates at low salt concentrations. Thus, there are important differences between the proteins in the seeds of two species of the same plant family, a fact which is worth further study. From the experimental results of the studies on the dissociation and association of the proteins in seeds of peanut, lupin, and soya bean some very important conclusions can be drawn. All of them yield practically monodisperse fractions at high ionic strengths. Where a mixture of components has been obtained, the separation experiments indicate that complete separation is possible. Dissociations and associations are observed when the salt concentration is lowered. It is possible that the proteins are changed in some way when the ionic strength has reached the limit where the proteins have a low solubility.

To learn more about this problem, the peanut proteins, especially the arachin fraction, should be reinvestigated at high ionic strengths along the same lines as used for pea and lupin proteins. In this way, the arachin could

be studied over a broader pH range. For the same reasons the pea proteins should be studied at low ionic strengths. The reviewer is of the opinion that monodisperse fractions should be isolated at high ionic strengths as the first stage in the study of the seed globulins. When the monodisperse fractions so obtained have been chemically characterized, i.e., by means of amino acid analysis, the more subtle dissociation and association reactions should be studied.

Isolation Experiments on Proteins from Various Plants

Squash seed proteins.—Crystalline seed globulins can easily be obtained from some cucurbitaceous plants. The methods of isolation were described by Osborne (48) and the crystalline proteins so prepared have repeatedly been used as model substances for the determinations of the amino acid contents of proteins. The work of the last decade has shown, however, that the fact that a protein can be obtained in crystalline form is no criterion of homogeneity. For this reason it is logical to study the homogeneity of the crystalline proteins with the new methods now available. Byerrum *et al.* (49) studied the globulin fraction from seeds of *Cucurbita pepo* electrophoretically at pH 4.0, and found one single protein component at this pH. A study of homogeneity must, however, be carried out at different pH values in order to give complete information. Fuerst *et al.* (50) investigated a similar protein both in the ultracentrifuge and with electrophoresis and obtained many important results. In their paper, references are given to earlier studies of these globulins. Fuerst *et al.* worked with seeds from squash, *Cucurbita maxima.* The ground seed material was extracted with 10 per cent NaCl at 40°C., and the extract was filtered and centrifuged. The clear extract was diluted fivefold with water at 50°C., and allowed to crystallize at 2°C. overnight. The precipitate was recrystallized three times by the same dilution method. The recrystallized protein, dissolved in 10 per cent NaCl at pH 5.9, gave only one component in the ultracentrifuge.

At low ionic strengths (0.1 *M* acetate or glycine buffer), the globulin was polydisperse, and in some cases four components were obtained. The authors showed that under the experimental conditions used the globulin fraction consists of a dissociating system. The main component has a sedimentation constant of 7.1 S. Fuerst *et al.* point out that some of their observations are not in line with their dissociation hypothesis. One important fact in this connection is that at pH 4.7 one component is obtained in the ultracentrifuge, whereas two components are found on electrophoresis. At pH 3.9 one electrophoretic omponent is obtained thus confirming the work of Byerrum *et al.* (49).

The methods of Fuerst & co-workers can, however, be seriously criticized from one important point of view. They work with recrystallized proteins which are dissolved in buffer solutions of low ionic strength and of different pH values. They comment on their method in the following way: "Aliquots of the crystals were dispersed in buffer solutions. Such dispersions were used

directly for sedimentation analysis unless dispersion was not complete. Undispersed material was removed by low-speed centrifuging." Such a method is not suitable when a dissociating protein system is studied. In the experiments described earlier in this review, e.g., by Johnson *et al.* and by Joubert, the proteins were first dissolved and then the ionic strength was varied. Many of the effects which were observed by Fuerst *et al.* may be understood by assuming that the crystalline globulin fraction consists of two components which have different solubilities at different pH values. The pH range in which the squash globulins are isoelectrically precipitated is very broad (4.7 to 8.3) because of the very low ionic strengths used in this study. If the experiments were carried out at a higher ionic strength, isoelectric precipitation might be a useful separation method for the two globulin components. The reviewer is of the opinion that this protein system should be reinvestigated in buffers of higher salt concentrations. The dissociation process should be studied by dialysing against buffer solutions of different ionic strengths according to Joubert (32) with a careful control to insure that no proteins are precipitated during the dialysis.

Naras seed proteins.—The proteins of another cucurbitaceous plant, *Acanthosicyos horrida*, have been studied by Joubert & Cooper (51, 52). Two proteins were isolated. The main component has a low solubility and a molecular weight of 343,000, as determined by a light-scattering method. To increase the solubility of this protein for an electrophoretic study, dodecylsulphate at a concentration of 1 per cent was added to a buffer of ionic strentgth 0.1. In this buffer the protein dissolves easily by complex formation, but is broken down by this treatment. The complex is electrophoretically homogeneous and has a molecular weight of about 88,000. The other protein component in naras seed is easily soluble and its molecular weight as determined by Joubert & Cooper is 58,000. It is reported to have a high homogeneity.

Sunflower seed proteins.—The problems which Osborne studied 60 years ago are now of current interest. As an illustrative example Joubert (53) recently published a paper on sunflower seed proteins. The preparation procedure of Osborne & Campbell (54) was used, i.e., extraction with 10 per cent NaCl and precipitation of the proteins with $(NH_4)_2SO_4$ at different degrees of saturation. Osborne & Campbell thought the globulin fraction from sunflower seeds to be homogeneous. Joubert showed that this opinion was incorrect, because he observed four components in the ultracentrifuge diagrams. The main component has a sedimentation constant of 11.7 S and it is obtained practically pure by precipitation of an extract with $(NH_4)_2SO_4$ between 22 to 30 per cent saturation. The molecular weight, determined by sedimentation and diffusion experiments is 343,000. One component with $s_{20} = 1.67$ S is precipitated in the range of 40 to 80 per cent saturation. It is polydisperse and has a molecular weight of about 19,000. It cannot be understood from Joubert's paper whether or not this fraction is of a globulin nature. Experiences with protein systems from other plants indicate, how-

ever, that this fraction might consist of albumin, which could easily be decided by dialysis of a solution to the salt free state. It is remarkable that Joubert did not purify the main component by reprecipitation at different pH values.

In this review, the results of some recent studies on protein systems from seeds of many different plants have been summed up. As already indicated, a striking fact is that the theories of Osborne are used in nearly all of these later investigations on plant proteins. In most cases, however, it has been shown that Osborne's preparations were nonhomogeneous, but it is futile to deny the fundamental importance of his work in this field of protein chemistry. Now is the time to develop more efficient separation procedures for plant proteins, instead of only using unmodified methods of Osborne. It is, of course, important to investigate the protein systems of plants which have not as yet been studied, but this kind of work only has the character of cataloguing. It is far more important to study a few protein systems very carefully both from a physico-chemical and purely chemical point of view.

THE EFFECT OF CLIMATE AND OTHER FACTORS ON THE COMPOSITION OF THE PLANT PROTEINS

The chemical composition of plant proteins used as food is very important in animal nutrition because of the scarceness of animal proteins. The nutritional physiologists are looking for suitable plant proteins which can substitute for animal proteins. The main problem is to find plant proteins with a high biological value, i.e., with a high content of essential amino acids. Every year new determinations of amino acid composition on bulk proteins from different plant sources are reported. The development of paper chromatography and the separation of amino acids on ion exchange columns have facilitated these studies.

The problem of whether different cultivation conditions have any effect on the chemical composition of the plant proteins has been studied in several places, above all in Russia. The variation in total protein content of plants cultivated in different climates, soils, etc. has mainly been studied. Klimenko (55) studied peas which normally grow in different parts of Europe, Africa, and the United States. Seeds from these peas were planted in two different places in Russia—Moldavia and Precarpatia, and the peas harvested in these places were studied. Total N, protein N, and extractable N were determined in H_2O, NaCl, and NaOH extracts. From these experiments, Klimenko concluded that the conditions of cultivation are of importance in relation to the protein content of the seeds. He obtained similar results in a study on chick-pea (56). Similar investigations have been carried out in the United States. Price *et al.* (57) found differences of 10 per cent in protein content in seeds of leguminous plants cultivated in different climates. Similar results were obtained by Shollenberger *et al.* (58) in experiments on wheat. Evans *et al.* (59) studied the proteins in Alaska peas grown in different places and treated with different fertilizers. They found that sulfur-containing

fertilizers increased the cystine content but lowered the protein content of the seeds.

It is difficult to draw any definite conclusions from these experiments because the proteins were not separated into any well-defined fractions prior to analysis. The experiments should be repeated on seeds of *Pisum sativum* or *Lupinus angustifolius*, for which separation methods have already been developed. From the ripe seeds, harvested from plants which have been cultivated under different conditions, the amino acid composition of the separated globulins should be determined. By these means, an answer could be obtained to the important question whether or not the composition of the reserve proteins is constant, or whether the climate and soil can have any effect on the composition. The amino acid composition of the nonprotein, dialysable fraction and of the albumin fraction should also be determined. In this way, a clearer picture could be obtained of those changes in protein composition which have been reported by several authors.

THE SEED PROTEINS DURING GERMINATION AND RIPENING

The breakdown of the well-defined seed proteins during germination and their synthesis during ripening have not been intensively studied with modern methods of analysis. A study of the intermediate products which are formed in these processes might give important knowledge of the architecture of these proteins.

Danielson (60) studied the breakdown of legumin, vicilin, and albumin in seeds of peas during germination. It was shown in the ultracentrifuge that the two globulins, legumin and vicilin, were digested at the same rate. In extracts of seeds germinated for different lengths of time, the contents of extractable N, dialysable N, nondialysable N, globulin N, and albumin N were determined. The well-defined globulins are broken down most intensively after 5 to 10 days of germination, i.e., when the new plant develops new leaves. During the breakdown of the globulins the amount of dialysable N strongly increases. The albumin fraction is broken down very slowly. These results clearly show that the globulins consist of reserve proteins which, during germination, are digested to low-molecular weight substances. The amino acids, peptides, etc. formed are used in the synthesis of proteins in the developing plant. These experiments could be expanded in several directions, e.g., the breakdown of the well-defined proteins could be studied by different means, such as electrophoresis and amino acids analysis. Of special interest would be an analysis of the intermediate products formed, especially the simpler peptides.

A similar investigation was carried out on the reserve proteins in peas during the ripening process (61, 62). The globulins are synthesized very rapidly in an early stage of ripening. It was shown by ultracentifugation that vicilin and legumin are synthesized at different rates, because the ratio of vicilin to legumin decreased as ripening proceeded. Of special interest is

the fact that ultracentrifugation showed globulin synthesis to occur via high-molecular weight intermediate products. These substances should be isolated and studied with other methods, especially electrophoresis.

Some of the results obtained on ripening pea seeds have been confirmed by Hyde (63). Both investigations show that the nitrogen in the very small, unripe seeds occurs in low-molecular weight substances. During ripening the low-molecular weight nitrogeneous substances are quantiatively transformed into proteins. Hyde also showed the presence of different amino acids in the nonprotein fraction at different stages of the ripening process. He used paper chromatography for these observations. Some simple peptides were also isolated. It is, however, difficult to decide from these studies what happens with the separate amino acids during the ripening of the seeds.

Kondo *et al.* (64) followed the proteins electrophoretically during the ripening of soya beans. The soya beans were extracted with 1.5 *N* NaCl at different stages of ripening and analyzed in the electrophoresis apparatus. Five components were observed and, as ripening proceeded, the amount of one of these components increased and the concentration of the other components decreased.

The investigations which have been carried out so far on the seed proteins during germination and ripening must be looked upon as highly preliminary, but they open a new, interesting field in the study of plant proteins. Seeds, especially from leguminous plants, are excellent material for the study of protein synthesis, because their protein content is high and the proteins are often well-defined. Large amounts of material can be obtained at different stages of development and can be stored in a frozen or freeze-dried state without undergoing any changes. In many ways seeds are more convenient for protein studies than is material from the animal kingdom, e.g., regenerating liver.

A special problem which has to be solved is whether or not the synthesis and breakdown of the plant proteins occur via well-defined intermediate products of high-molecular or low-molecular weight. If such intermediates could be isolated, it should be determined whether they are the same during the breakdown and the synthesis of the proteins. This problem is of great importance for enzyme chemistry because it can answer the question of whether or not the hydrolyzing and synthesizing proteolytic enzymes react at the same points of the polypeptide chain. For a study of these problems, a combination of chromatographic and tracer techniques probably must be used.

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PHYSIOLOGY OF ROOT ELONGATION[1,2]

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Root elongation is the result of the enlargement of new cells constantly being formed by cell divisions in the general region of the apical meristem. Subsequent differentiation of these cellular components of the root gives rise to the complex tissue systems which comprise the mature structure. Secondary branching with the formation of new meristems of lateral roots as well as secondary thickening resulting from the initiation of a vascular cambium further complicate the picture of root development. In reviewing the recent work on the physiology of root development, it has become increasingly clear that there is an urgent need to distinguish in experimental studies between the respective contributions of cell division, cell enlargement, and cell maturation. It is proposed in this review to restrict the discussion largely to the process of root elongation which, as pointed out in Burström's review of this subject (48), is, in itself, a complex phenomenon. An attempt will be made to summarize some of the pertinent results of the large amount of recent work centering about the phenomenon of root elongation and its physiological control.

The Measurement of Root Elongation

Root growth has been defined and measured by different workers in many diverse ways, and thus the term growth in reference to roots has lost precise meaning. It therefore becomes important in comparing results and assessing conclusions to appreciate differences in methods of measurement and the actual processes studied. Root elongation can be equated unambiguously to total increment in root length and is most directly measured by changes in linear dimensions. Root elongation results from the combined effects of mitotic activity of root cells and the enlargement of cellular components in the direction of the longitudinal axis. Root elongation measurements are the most frequently made because of the ease and speed of measurement and have been especially used in testing physiological activity of growth regulating substances. Root elongation tests based on measurement of length of intact seedling roots include tests developed by Swanson (136), corn (*Zea mays*); Ready & Grant (116), cucumber (*Cucumis sativus*); Moewus (96), cress (*Lepidium sativum*); Åberg (1), flax (*Linum usitatissimum*); Leaper & Bishop (87), lupine (*Lupinus albus*); Audus (16), cress (*Lepidium*

[1] The survey of literature pertaining to this review was concluded in October, 1955.

[2] The following abbreviations will be used: IAA, indoleacetic acid; NAA, α-naphthaleneacetic acid; IAN, indoleacetonitrile; 2,4-D, 2,4-dichlorophenoxyacetic acid; DNP, 2,4-dinitrophenol; TIBA, 2,3,5- triiodobenzoic acid; PCIB, *p*-chlorophenoxyisobutyric acid; NMSP, α-(1-naphthyl-methyl-sulfide) propionic acid.

sativum); Goodwin & Taves (67), oats (*Avena sativa*); Ashby (14), artemisia (*Artemisia absinthium*); Pilet (105), lentil (*Lens culinaris*); San Antonio (122), sweetclover (*Melilotus alba*); and many others. Results of such tests are inevitably difficult to interpret in cellular terms unless measurements relating to cell changes accompany the total length measurement. Difficulties arising from use of such tests in determining auxin activities will be discussed subsequently.

In attempts to relate root elongation to cellular processes, direct microscopic measurements of changes in cell length have been made by a number of workers. Measurements of epidermal cell lengths in the elongating portion of wheat seedling roots have been used as criteria of root elongation by Burström (38 to 51) in an extensive series of papers beginning about 1942. A similar procedure was used by Brumfield (37) in studying seedling roots of *Phleum* and was one basis for the analysis of root growth in *Phleum* by Goodwin & Stepka (66). More recently Lexander (91) has devised a rapid 24 hr. test for measuring the effects of applied substances on epidermal cell elongation in wheat roots. It has been used by him to test for auxin activity in plant extracts and by Burström (50) to study antiauxin activity in root elongation. In his studies of root elongation in roots of *Zea mays*, Baldovinos (22) measured elongation of cortical cells, which he selected as representing the major tissue component of the elongating roots. These measurements together with counts of mitotic figures in the cortical tissues were made the basis for calculating rates of cell division and enlargement in the growing roots.

It is the general assumption of such work that length changes in the selected cells are typical of changes of all cells and thus of root elongation. The limitations of the exclusive use of this method in studying root growth have been pointed out by Brown *et al.* (33). Wanner (146) measured cell lengths of epidermal, cortical, and central cylinder tissues and found them to begin elongation quite out of phase with each other. Burström (45) has clearly shown that increase in root length need not be reflected in corresponding increase in epidermal cell length, which will vary with the nutrient medium—e.g., the relative supply of nitrate and phosphate in the medium. Street & McGregor (134) have found in excised tomato roots no direct correlation between epidermal cell length and total root length when roots are grown in different concentrations of sucrose. And in the presence of certain types of growth regulating substances, it has been shown that epidermal cells behave quite unlike the other tissues of the root [Burström (39), Sandström (123)]. Furthermore, epidermal cells of *Phleum* (66) and of roots of many plants [Cormack (55)] are of two distinct types of quite different lengths with one type fated to become root hair cells. As Burström (39) has indicated, the striking difference in response between epidermal cells and cortical parenchyma cells to diamylacetic acid indicates a "remarkable difference in the sensitivity of the cell layers." Such differences in sensitivities of the tissues of the root to diamylacetic acid, to 2,4-D [Wilde (148), Kandler

(78)], and to other growth regulators, point up the dangers of using cell length as an exclusive measure of root growth. It is to be expected that cell-length measurements may be related to root elongation only when such a correlation is shown to exist. To assume such a relationship, especially in tests of externally supplied auxins (or auxin antagonists), may lead to serious errors.

Another method of studying root growth has been applied by Audus & Shipton (20) and by Audus & Thresh (21). Their method involves the determination of increases in fresh weight over a period of one to two days of 2 mm. sections excised from the "extending zone" of pea roots. The increase in fresh weight is termed "growth," and the method, used to measure the effect of synthetic auxins and of plant extracts on root growth, is claimed to be more sensitive than straight growth tests using *Avena* coleoptile sections. The authors (20) equate this measure of growth directly to "extension growth" since they state there is a negligible increase in diameter of the sections and most of the increase in fresh weight is thus attributable to extension along the main axis.

It had been shown earlier in roots of *Zea mays* [Brown & Sutcliffe (35)] that the segment 1.5 to 3.0 mm. behind the tip showed vigorous extension after excision and was devoid of cellular divisions as determined by cytological study and cell counts. Elongation of such segments is directly attributable to cell enlargement and is most closely analogous to the peculiar and distinctive situation of extension growth shown to exist and studied so extensively in *Avena* coleoptile sections. Audus & Shipton do not state whether they made an analysis for cell divisions in control pea root segments used in their test, although such analysis would be desirable. Volume measurements alone, unless checked at each treatment with section-length measurements, would not necessarily differentiate between the effects of applied substances on cell enlargement in the direction of root elongation in contrast to enlargement in the radial direction. The latter is a common response of root tissues to auxins and certain growth inhibitors [Levan (90), San Antonio (122), Pollock *et al.* (114)]. Nonetheless, the method devised by Brown & Sutcliffe and used by Audus & Shipton comes close to meeting the requirements of a sensitive root growth test, which measures exclusively cellular enlargment of root cells. For purposes of testing plant extracts and unknown concentrations of auxin, this test has its limitations, some of which are pointed out by the latter authors. Using the same method, Åberg & Jönsson (8) have shown that responses of root sections to applied growth regulating substances are quite different from those of intact seedling roots, a fact apparently related to the exposure of cut surfaces and greatly increased oxidative activity.

With increasing interest in isolated root culture as a means of studying root physiology, there have been a number of studies of root elongation and its inhibition, using cultured clonal root materials. The root, excised from the germinating seed or the intact plant, can be expected to be quite different

in its physiology from the intact seedling root, but elongation measurements still represent the resultant of the two cellular processes of multiplication and enlargement. Development in excised roots is inherently no less complex than in intact roots.

Boll (26, 27) has measured growth in isolated tomato roots in terms of increase in length of the main axis, the number of laterals per root, and the total length of ten basal laterals per root. Growth measured in such terms is highly complex, and resulting data are manifestly difficult to interpret either in cellular, physiological, or biochemical terms. In nutritional studies which have as their goal the achievement of maximum root development in a completely known synthetic medium, measurements of this sort have proved of value [White (147)]. For such studies, fresh and dry weight determinations, together with linear measurements may be useful (133, 134). Kandler and his colleagues (78 to 81) and Zöttl (149) have studied the influence of a number of synthetic plant regulators on assimilation in isolated roots of *Zea mays* in culture. Measurements included length, dry weight, total nitrogen content, respiratory activity, and glucose utilization. Direct conclusions concerning growth processes which are referable to cellular processes are likewise difficult to achieve from such studies, which serve more to illustrate the complexities involved and to direct attention to aspects suitable for detailed investigation. The physiological and biochemical information thus derived may ultimately be of value in interpreting, for example, the process of cell enlargement.

Schopfer and his colleagues (124 to 127) have studied the growth of isolated pea roots in synthetic nutrient medium and the effect on root elongation of a series of growth factors and growth antagonists. With certain substances, e.g., the action of homothiamine-glycol, growth inhibition could be attributed specifically to cessation of cell divsion; with others, the nature of the effect was not easily explicable in cellular terms.

In a study concentrating on cellular activities in root growth, Brown & Wightman (36) analyzed root lengths of isolated first-transfer pea roots grown in nutrient medium by determining cell numbers by direct count. Their analysis showed that in excised root tips of different initial lengths, length increment is closely dependent upon the rate of increase in the total number of cells and that this rate increases with time. Their results emphasize the important role of cell division in determining root elongation and suggest that continued meristematic activity of the root tip is dependent upon materials translocated toward the tip from the mature regions of the root.

In still other studies using isolated roots grown in culture, Street (131, 132) and Street *et al.* (135) studied root growth by recording increase in main axis length and root survival, i.e., continued meristematic activity, in repeatedly subcultured excised tomato roots. They have tested the effects of a number of growth regulators on root growth and by these *in vitro* culture methods claim to be able to analyze the effects of internal and external

factors determining cellular division and cell enlargement in growing roots. The method has the advantage that growth of isolated roots depends completely on the nutrient medium provided and the inherent synthetic capacities of the root tissues themselves. Torrey (143) has pointed out the advantage of extremely short root tips (1 mm. or less) for studies of root growth *in vitro* since such tips are readily depleted of their externally supplied nutrients. Cessation of cell divisions in such tips is reflected in almost immediate cessation of root elongation.

As is evident from the above discussion of methods currently used in studying root growth, precise separation of the different aspects of root growth—i.e., by cell multiplication and by cell elongation—has been very difficult to achieve experimentally; and yet it is precisely this separation which is essential to an understanding of root elongation in terms of physiological mechanisms, a fact which has been emphasized recently by Hansen (71). Only when analysis of root elongation is possible in terms of constituent cellular processes will our detailed understanding of mechanisms be furthered. It is important to appreciate that only in rare cases is it possible to measure directly root growth which results wholly from cell enlargement; rather, in the usual situation, cell enlargement, cell multiplication, and cellular differentiation are inextricably interrelated.

In recent years a number of papers have been published which serve to define more clearly the problems inherent in analyzing root elongation. The general concept is pressed that an understanding of root elongation must be based upon knowledge of the nature, activities, and fate of all the cellular components; and it is further conceived that the differentiation of cells in linear sequence along the longitudinal axis represents a physiological gradient which can be analyzed and interpreted. In a series of papers, Brown *et al.* (31 to 36) in the period from 1950 to 1955 have endeavored to present an integrated interpretation of normal root development in terms of cellular activities. Using untreated roots or root segments taken from intact seedlings of pea, Brown & Broadbent (31) determined changes in volume, dry weight, total and soluble nitrogen content, and respiration per average cell during the process of cell enlargement. Cell number determinations were made by cell counts of macerated root segments of known dimensions. Their data, calculated on an average cell basis, represent changes in physiological processes which occur during root elongation and are subject to interpretation in cellular terms. Using the same rather elaborate technique with roots of broad bean (*Vicia faba*), Robinson & Brown (119) attempted to correlate physiological changes in root cells during root elongation with changes in specific enzymatic activities. In a later paper (120), these authors made further determinations of enzyme activities and their changes during the growth of roots. Morgan & Reith (98) followed changes in amino acid composition and total changes in protein, peptide, and free amino acid content of the same bean root material and were able to express these changes on a per cell basis. Brown, Reith & Robinson (33) and Brown & Robinson (34),

in reviewing the results of their work, have proposed the outlines of a general scheme for cell enlargement during root growth based on a concept of continuous change in the pattern of enzymatic activities of the cell. They believe that cessation of cell enlargement is attributable to some "arresting mechanism" which may be a part of the changing enzymatic activity of the cells. While not providing answers as to the details of the growth mechanism, this approach to the problem of root growth has provided us with a fairly intimate knowledge of some of the manifold cellular changes upon which growth depends, and points the direction in which more detailed analytical work must proceed.

Recently, Jensen (76), working with 200 μ thick serial sections of *Vicia faba* roots, reported still further refinements of technique which permitted him to correlate morphological and biochemical information on a cellular level in which the diversity of cell types in the growing root may be taken into account. With the advent and use of such experimental approaches, it becomes clearly possible to distinguish the various aspects of root growth and to study more specifically the component processes of root elongation.

The Inhibition of Root Elongation

The elongating root is a highly organized and actively metabolizing system which is directly affected by the surrounding physical and chemical environment. Because of its sensitivity, the inhibition of root elongation has been used extensively in the study of biological activity of chemical compounds [e.g. (52)]. Most such inhibition studies are based on the simple procedure of determining mean total length at the end of a given time period, expressed as a percentage of the control. As a method for initial screening of large numbers of compounds for "biological activity" which are later to be investigated in detail, the procedure has its usefulness. As a method for the study of the processes of root elongation *per se*, it is highly limited, and limitations, such as those already discussed, must be recognized.

Audus (17) has pointed out the danger of a "total growth" determination as the exclusive measure of growth inhibition, without following the time course of the inhibition. Quite different conclusions may be reached when growth rate rather than total growth is used. Audus has shown, for example, the importance in inhibition studies of the rate of penetration of the potential inhibitor, a factor which can be completely missed in total growth determinations. Furthermore, growth rate is usually a much more sensitive measure of inhibition than a total growth determination. The desirability of using time course studies in following growth phenomena has been pointed out by many authors, and elegant methods have been devised and are being applied to root inhibition studies [e.g., Erickson & Goddard (60), Goodwin & Pollock (65)].

Inhibition of root elongation may, of course, be caused by a wide variety of substances. This lack of specificity had led to serious questions concerning the usefulness of elongation as a measure of any specific type of activity

as, for example, auxin activity. It is impossible to ascribe inhibition of root elongation by applied compounds to inhibition of specific physiological processes concerned with cellular multiplication and cellular division unless detailed analyses of the cellular changes accompany the simple length measurement. Inhibitors of cell division as surely cause cessation of root elongation as do inhibitors of cellular enlargement. Thus, in inhibition studies, as in studies of normal root elongation, analysis of the elongation process reduces itself to a consideration of cellular processes and cellular changes which must be examined both at the cellular and at the biochemical level. It is unfortunately true that in most of the published work on root inhibition, there is no evidence concerning changes either in cell number, cell size, or cellular differentiation during the period of inhibition, and it is thus impossible to interpret the results in a meaningful way, so far as understanding the phenomenon in cellular terms is concerned. It is true that in some cases one can interpret the inhibitive effect in biochemical terms directly, but without the possibility of relating the biochemical mechanism of control to cellular activities.

Certain studies of root inhibition in cellular terms have been made. Burström, in his work over the years on the auxin inhibition of wheat root elongation (38 to 51), has analyzed in detail many of the cellular properties of inhibited roots and is in a position to discuss certain aspects of the inhibition in cellular terms. Using cell counting procedures following maceration of treated roots of *Cucurbita pepo*, Hopkins (73) studied the inhibition of root elongation by benzene hexachloride isomers and certain enzyme inhibitors. Striking differences are evident in the effects of the different inhibitors of root elongation. For example, 2,4-dinitrophenol (DNP) at $4.2\times10^{-6}M$ (pH 6.0), while having no effect on total root length, caused a decrease of 30 per cent in the mean total number of cells per root, an obvious effect on cell multiplication which was not evident in the length measurement. Sodium arsenate at 2.5×10^{-6} M (pH 6.0), while having no effect on cell multiplication, reduced mean root length by approximately 20 per cent, an effect at this concentration apparently restricted to the process of cell enlargement.

Torrey (142) on the basis of histological studies of inhibited roots, observed the effect of metabolic inhibitors on vascular tissue differentiation. Using concentrations which produced 90 per cent inhibition of pea root elongation in nutrient culture, he found that whereas DNP completely inhibits vascular tissue differentiation, IAA markedly accelerates certain aspects of such tissue differentiation. With inhibition of elongation by IAA, cellular divisions in lateral meristems were accelerated, whereas comparable inhibition of root elongation by iodoacetate completely inhibited cell multiplication. Here, again, extensive differences in response at the cellular level were apparent which were not reflected in the simple measure of inhibition of root length.

Pollock *et al.* (114), using methods which allowed careful microscopic examination of cellular changes as well as the progress of inhibition with

time, were able to distinguish between the effects of compounds which inhibited elongation by upsetting the direction of cell enlargement and those which prevented cell multiplication.

Metabolic inhibitors.—Known inhibitors of specific metabolic processes have been used extensively (28, 139) in the study of *Avena* coleoptile section growth in attempts to relate the phenomenon of auxin-controlled cell enlargement to specific respiratory reactions. Many metabolic inhibitors have been reported effective in root inhibition at about the same range of concentration as for inhibition of shoot cell elongation or of metabolic activities of living tissues in general [Albaum & Commoner (9), Henderson & Stauffer (72)]. Hopkins (73) reported the effects of a large number of known metabolic inhibitors. The most striking inhibitions of both cell multiplication and total root length were caused by DNP and sodium arsenate, both agents thought to act by uncoupling oxidative phosphorylation. Beryllium sulfate, believed to be an inhibitor of adenosinetriphosphatase, markedly inhibited root elongation without any apparent effect on the growth index, indicating the inhibition was restricted to cellular enlargement. Sodium arsenite and iodoacetate, inhibitors of sulfhydryl enzymes, were less effective in inhibiting root elongation. Sodium fluoride was reported to have no effect on root growth over the range 10^{-6} to 10^{-2} *M* at pH 6.0. Cooil (54) was unable to find inhibition of *Avena* seedling root elongation in the presence of 2×10^{-5} *M* iodoacetate (pH 6.0). Kandler (77) reported that DNP at 10^{-5} *M* effectively inhibited root elongation of excised roots of *Zea mays* although at 1.7×10^{-6} *M* (pH 5.5) the same compound stimulated root elongation for up to eight days, followed by a slight inhibition. Kandler (78) also studied the effect of several inhibitors including 2,4-D, NAA, and sodium fluoride on elongation of isolated *Zea* roots cultured for five days. Fifty per cent inhibition of root elongation was caused by sodium fluoride only at concentrations greater than 2×10^{-3} *M*, while a slight stimulation of root elongation was apparent at 10^{-4} *M*. It is interesting that the addition of thiamine to the medium at high concentrations, i.e. 10^{-2} *M*, produced greater than 20 per cent inhibition while stimulation of root elongation was noted at concentrations of 3×10^{-3} *M* and below. Kandler reported changes in dry weight, nitrogen content, glucose utilization, oxygen utilization and respiratory quotient during the growth periods. The consistent effectiveness of DNP in causing inhibition of root elongation is in agreement with the observation of Bonner (28) on DNP inhibition of cell elongation in the *Avena* coleoptile, suggesting that utilization of high energy phosphate is of primary importance during root elongation.

Pohl (110) has pointed out that the typical growth response to inhibitors of root elongation, including auxins, shows the course of a sigmoid curve which, when expressed as a logarithmic plot, is linear over the inhibitory range. The inhibition response, according to Pohl, is independent and quite distinct from the optimum curve which characterizes the stimulatory response to low concentrations of inhibitors. This separation of responses,

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he feels, is particularly important in interpreting the effects of auxins on root elongation, a matter to be discussed shortly. In the course of analyzing the auxin inhibition response, Pohl & Ochs (113) tested several metabolic inhibitors on root elongation in the cress root test. Concentrations of 5×10^{-4} *M* iodoacetate and 10^{-3} *M* potassium cyanide (pH not specified) caused essentially no inhibition of root elongation, whereas DNP produced approximately 50 per cent inhibition at 5×10^{-5} *M*. The latter figure agrees well with concentrations discussed earlier with respect to the particularly strong inhibitory effect of DNP. In tests with these inhibitors in the presence of indoleacetic acid, Pohl & Ochs report that noninhibitory concentrations of iodoacetate and DNP, but not KCN, interfere with inhibition of root growth by high auxin levels. The stimulatory effect on root elongation of low auxin concentrations is reduced by DNP and KCN, but is unaffected by iodoacetate. In view of the possibility that the differences in activity of these inhibitors may be related to pH differences which were not controlled, it is desirable that these effects be confirmed under carefully specified conditions.

The authors believe these results support their contention that the stimulation caused by auxins in low concentration is a reaction quite independent of the inhibitory effect of auxin at higher concentrations. Over the inhibitory range, auxin is believed to interfere directly in respiratory processes, while at low concentrations auxin action is thought to involve adsorption on the lipoidal film of the cytoplasmic boundary of the cell without direct effect on respiratory reactions of the cell. Pohl (111, 112), in applying these ideas to shoot elongation, compares the logarithmic response in the root (auxin inhibition) to the logarithmic response of coleoptile section elongation (auxin stimulation). In the former, metabolic inhibitors counteract the auxin inhibition of root elongation, whereas in the latter system these same inhibitors prevent the auxin stimulation of cell elongation. Pohl considers the two cases to involve the same reaction system. From the work of Hopkins (73) discussed above, it is manifestly difficult to compare inhibitor effects on a system, such as the root, in which cell division and cell elongation both contribute significantly to the total length measurement with those produced in a system in which cell enlargement alone affects length measurements. If the assumption is made with Pohl that the reaction system is the same in the shoot and the root, a hypothesis which is far from established, one is still faced with the fundamental problem concerning the remarkable difference in the sensitivities of the two systems to externally supplied auxin.

The marked dependence of normal root elongation on the pH of the external solution has been discussed by Burström (48). As Burström has pointed out, no agreement exists as to the nature of the effect on root growth of changes in hydrogen ion concentration of the external medium but there is no doubt that pH has marked effects on root growth and must be controlled if experiments are to be meaningful. The importance of pH becomes

particularly evident in the study of the effects of inhibitors on root elongation and has been discussed by Audus (15). Effectiveness of inhibition by a given reagent may depend almost completely on the pH at which the test is made. Disagreement in the literature as to inhibitor effects on root elongation may be due in large measure to lack of pH control.

There is considerable direct evidence that root elongation, especially the cell enlargement phase, is dependent upon certain metabolic processes which would clearly be interfered with by specific metabolic inhibitors. Certain studies have suggested that root elongation may be limited or influenced by the rate of cellulose synthesis in the wall. Brown & Sutcliffe (35), analyzing chemical changes in elongating root segments of *Zea* in nutrient medium, showed that segment elongation was dependent upon externally supplied sugar. With an increasing supply of sugar there occurred a related increase in cellulose formation. Their conclusion, that the rate of extension depends on the rate of cellulose synthesis, does not agree with data of Jensen (76), who found in seedling roots of *Vicia faba* that cellulose formation actually ceases temporarily as cell elongation begins. Jensen finds, rather, a steady increase during cell elongation of an unidentified nonglucose carbohydrate while the cellulose content remains constant. Karlsson & Eliasson (82), on the basis of respiratory quotient determinations on elongating wheat root segments, suggested that in the absence of substrate, cell wall dissolution may actually occur in the extension region with the utilization of the released organic products as respiratory substrates. Whereas Brown & Broadbent (31) found an increase in protein content during root cell elongation, Jensen (76) reported a cessation of protein synthesis as cell elongation begins and an actual decrease in protein content during cell elongation. Both authors reported increased respiratory activity on a cell basis. Wanner (146) and others (59, 85) have also reported high oxygen uptake per unit of total nitrogen in the region of most rapid cell elongation. The findings of Ranson & Parija (115), that root elongation is closely correlated with the available oxygen supply, also tend to confirm the importance of aerobic respiration for the process of cell elongation. Although there is conflicting evidence as to specific changes in cellular constituents, there appears to be general agreement that rapidly elongating cells show high metabolic activity and that such metabolic activity in some way determines the behavior of the cell wall during its extension. A metabolic inhibitor, such as DNP, which blocks a crucial step in this metabolism, may be expected to stop cell enlargement and root elongation.

In summarizing the above results concerning the effects of metabolic inhibitors on root elongation the following points are evident: first, root elongation is dependent upon cellular metabolism including at least aerobic respiration and concomitant phosphorylation; metabolic inhibitors can prevent elongation by inhibition of cell division or cell enlargement; the specific effects of individual inhibitors remain to be determined. It is clearly

desirable that detailed analyses of inhibition of root elongation be studied in cellular terms, using methods such as those of Brown *et al.*, Jensen, and others discussed above.

Inhibition by unsaturated lactones.—Another group of nonauxin inhibitors which has received considerable attention with respect to root growth is the unsaturated lactones. There appears to be no agreement as to the mechanism of action of these inhibitors. In tests of a wide variety of coumarin derivatives, Goodwin & Taves (67) found a number of compounds which markedly inhibited *Avena* root elongation. Especially effective was coumarin itself which at 10^{-3} *M* showed progressively greater inhibiting effects with time, leading to complete inhibition. At 3×10^{-5} *M*, although causing an initial inhibition of about 50 per cent, coumarin became progressively less effective. Coumarin is normally found in the roots of *Melilotus alba*, and it has been suggested speculatively by San Antonio (122) that these roots, which are readily inhibited by externally applied coumarin, may show an internal inhibition attributable to the natural compound.

Scopoletin (6-methoxy-7-hydroxy coumarin), a naturally-occurring unsaturated lactone present in *Avena* roots [Goodwin & Pollock (65)], is effective as an inhibitor of root elongation at concentrations somewhat less than those actually present in the mature portions of the root. Pollock *et al.* (114) have given evidence that scopoletin may play some role in the normal growth and differentiation of *Avena* roots. Coumarin was found to be 50 times as effective as scopoletin in inhibition of root elongation; it upset the polarity of cell elongation without stopping cellular division in the meristem. Scopoletin, on the other hand, appeared to inhibit all phases of the growth process. These authors were unable to relieve root inhibition caused by the unsaturated lactones by the addition of 2,3-dimercaptopropanol (BAL), a compound known to protect sulfhydryl enzymes from inactivation. They found also that inhibition of root elongation resulting from added indoleacetic acid was enhanced by the addition of either scopoletin or umbelliferone.

Andreae (11) reported inhibition of root elongation following soaking of pea seed in 2.5×10^{-4} *M* scopoletin whereas stimulation of root elongation occurred at 5.2×10^{-6} *M*. It was suggested that scopoletin, acting as a competitive inhibitor of IAA oxidation, was functioning via an auxin-sparing mechanism. Andreae & Andreae (12) showed further that methyl umbelliferone (4-methyl-7-hydroxy coumarin), was a highly effective inhibitor of root elongation in cress seedlings at 2.5×10^{-4} *M*, but, unlike scopoletin, actually stimulated IAA oxidation. They concluded that the IAA oxidase caused excessive auxin-destruction leading to inhibition of root elongation. Coumarin itself was found to have no effect on IAA oxidation (11), and its inhibitory action was presumed to be of a different character. Maleic hydrazide and 2,4-D were both shown to stimulate IAA oxidation *in vitro*, and these stimulations could be largely overcome by scopoletin (12). Their data on the difference in activity of methyl umbelliferone and scopoletin do

not appear to agree with the observations of Pollock *et al.* (114), who found both compounds to increase the inhibitory action of IAA on root growth when they were applied with the auxin.

Burström (50) reported the effects of coumarin and daphnetin (7,8-dihydroxy coumarin) on root elongation in wheat seedlings. Measuring total growth after 24 hr., Burström found that coumarin at 10^{-4} *M* caused complete inhibition of root elongation, at 5×10^{-5} *M* approximately 50 per cent inhibition, but no stimulation at lower concentrations. He called the inhibition an "all-or-none reaction" related to the effect of coumarin in preventing dissolution of the cell wall. Daphnetin, on the other hand, at 10^{-3} *M* produced complete inhibition, at 5×10^{-3} *M* 50 per cent inhibition, and at 10^{-4} *M* greater than 50 per cent stimulation of root elongation. The activity of daphnetin was believed to be comparable in nature to that of coumarin but only one-tenth as great. Burström interpreted the effects of coumarin-type inhibitors in terms of his two phase concept of the mechanism of cell enlargement.

Inhibition by antimetabolites.—Still another group of compounds shown to inhibit root elongation are the antimetabolites. These compounds are structural analogues of vitamins or growth factors required in normal metabolic activities during root growth. The competitive inhibition of a physiological process by an antimetabolite is generally considered to be good evidence of the activity of the metabolite in the process studied. Relief of the inhibition upon addition of the metabolite is confirmatory evidence concerning the biochemical site of the inhibitory action.

Antimetabolites at relatively low concentration may profoundly affect root development, including total root length. The inhibition of root elongation by sulfonamides and its reversal by *p*-aminobenzoic acid have been the subject of a number of studies [see Anker (13)], leading to the general conclusion that *p*-aminobenzoic acid is a normal metabolite in root tissues and that its function is essential to normal root elongation. Recently Boll (27) reported approximately 50 per cent inhibition of increase in main axis length of excised tomato roots by sulfanilamide at 2×10^{-5} *M* which was overcome by the addition to the medium of low levels of *p*-aminobenzoic acid. Similarly, Boll (26) has shown that 50 per cent inhibition of increase in main axis length in excised tomato roots was caused by approximately 1.5×10^{-5} *M* desoxypyridoxin which could be relieved by addition of pyridoxin, suggesting the inhibition was one of a competitive nature. Using excised pea roots grown *in vitro*, Schopfer *et al.* (125) found that 3×10^{-6} *M* homothiamine inhibits root elongation approximately 50 per cent and can be reversed by small amounts of added thiamine. Schopfer & Grob (126) have shown that 2,4-diamino-9,10-dimethyl-pteroylglutamic acid at similar low concentrations inhibits root elongation by blocking normal cell division. Its action is presumed to be an antagonism of normal pteroylglutamic acid function.

Fries (61, 62) found that hydroxyproline at 10^{-4} *M* caused approximately

50 per cent inhibition of root elongation of decotyledonized pea seedlings, an inhibition which could be partially overcome by L-proline at 10^{-3} *M*. Similarly, canavanine, an antagonist of arginine, at 10^{-4} *M* caused 50 per cent root inhibition, which could be reversed only slightly by added arginine. Azaguanine, a purine antagonist, at 10^{-5} *M* reduced root elongation to 50 per cent of the controls. This inhibition could be prevented by equimolar supplements of either guanine or adenine.

From these and related studies it is apparent that antimetabolites can be used as specific inhibitors in studies of root development. None of these studies has gone beyond the demonstration that certain metabolites or growth factors are normally utilized during root growth, a fact that might have been inferred from our existing knowledge of comparative biochemistry. It has not been possible thus far in plant materials to establish clear-cut symptoms of vitamin deficiences by the use of antimetabolites in a manner analogous to work on animals—which, if possible, might be a key to the specific role of the factor in plant growth or morphogenesis.

Natural inhibitors.—There is increasing evidence for the existence within roots of naturally-occurring compounds which are inhibitory to root elongation, but no clear indication of their nature or role in normal root elongation has been achieved. Vogt (144) found that ethanol extracts of rye (*Secale*), barley (*Hordeum*), or wheat (*Triticum*) roots are markedly inhibitory to root elongation of seedlings of rye or *Lepidium*. On dilution, the extracts caused slight stimulation of root elongation. Lexander (91), using chromatographic separation of ether extracts of wheat roots, reported the presence in the extracts of a substance believed to be IAA, a second substance which is stimulatory to both root and shoot elongation, and a third substance inhibitory to both processes. Bennet-Clark & Kefford (23) and Kefford (83, 84), using chromatographic methods, reported IAA to be present in roots of *Vicia faba*, *Pisum sativum*, and *Zea mays*. They have shown, in addition, that ether extraction of these same plant materials removes an accelerator (α) and an inhibitor (β) affecting both root and shoot elongation. The accelerator has considerable stimulatory effect on root elongation at physiological concentrations but becomes inhibitory at high concentration. It has been suggested by Stowe & Thimann (129) that accelerator (α) is indolepyruvic acid. The inhibitor, even at dilute concentrations, shows only growth-inhibiting effects on both root and shoot tissues. Howell (74) has contributed indirect evidence for the existence in roots of *Pisum* of an ether-soluble inhibitor of shoot elongation. Burström (50) has also assumed the existence of some unknown inhibitor of root elongation which he refers to as "factor X."

Libbert (92, 93) postulated that a natural growth inhibitor active in plant tissues must be released by auxin action from an inactive precursor originating in the root. The inhibitor occurs in the neutral fraction of ether extracts of roots of *Pisum*. Libbert proposes that in roots low auxin concentrations antagonize the action of the inhibitor, causing stimulation of

root elongation, whereas high auxin concentrations stimulate the conversion of the precursor to inhibitor thus producing root inhibition. The experimental evidence offered in support of this hypothesis is slight, although some details of the chemical properties of precursor and inhibitor have been reported (93). The study of natural inhibitors and accelerators, which has been so facilitated by the use of paper chromatography, may lead to a better understanding of the apparently numerous internal factors controlling root elongation.

Auxins and Root Elongation

Auxin inhibition.—The sensitivity of roots to externally applied auxin solutions is well known. The inhibition of root elongation at relatively high auxin concentrations and the slight stimulation of root elongation at low concentrations have been fitted into a general picture of plant response to auxins by Thimann (138). He pointed out that the response of roots to auxin over a wide range of concentrations shows an optimum curve which parallels the growth response shown by stem tissues, the latter having an optimum at a much higher auxin concentration than the former. This parallelism in response, while not explaining the response, places the observed effects of auxin in different tissues on a comparable basis. In recent years, the reality of the optimum curve response has been questioned with respect to root growth [Åberg (7)], as has the interpretation of the observed response [Pohl (111)].

There is no disagreement in the extensive current literature concerning the reality of the inhibition of root elongation by applied auxins, although there appears to be a considerable range in the sensitivity of roots to auxins. Depending upon the species and the conditions under which the test is run, 50 per cent inhibition of root elongation may be caused by auxin concentrations as low as 10^{-8} *M* (1) or only after the auxin level reaches 10^{-4} *M* (95). Thus, standardization of test conditions is essential if reproducible results using any one test method are to be obtained. In general, quantitative differences in responses of roots to auxin inhibition (or stimulation) which have been reported have been attributed to either external causes, i.e., environmental conditions of the experiment, or to internal causes related to the auxin physiology of the root itself. Awareness of the multitudinous factors influencing root response is essential if observed effects are to be interpreted correctly.

The sensitivity of roots to auxin inhibition has been shown to fluctuate widely with changes in the environmental conditions of the experiment. Åberg (1) devised two test methods for auxin inhibition, using seedling roots of flax. The tests differed in duration, temperature, and degree of submersion of the roots in the test solution. Åberg reported his "S-test" with roots grown in the test solution about 20 times more sensitive than the "F-test" in which seedlings grew on moist filter paper. As is suggested by this result, the availability and rate of absorption of the test compound, aeration of the

solution, temperature, and duration of the test may all be important variables influencing root elongation. The pH of the test solution has long been recognized to be of importance in determining the effectiveness of auxin on root elongation, although there is no agreement as to the nature of this effect [see Burström (48)]. Burström (46, 49) has shown the striking effect of cations, especially the calcium ion, on root elongation. Bosemark (30) reported that the response of wheat roots to externally-supplied auxins or antiauxins depends upon the supply of nitrate to the roots. Reinert (117) pointed out in particular the difficulties of using root inhibition tests for determining the auxin activity of plant extracts and like materials because of the masking effects of acids, salts, organic compounds, and other potential contaminants extracted with the auxin. In the recent report of a pea root test for determining auxin levels [Leopold & Guernsey (89), Leopold (88)], it is evident from the published experimental data that careful control of the environmental conditions for root elongation was not achieved and that the test cannot be considered reliable.

In addition to the influence of external conditions on root response to auxins, there is considerable evidence that the inherent physiology of the root itself controls the response to auxin and that roots differ widely from each other in this respect. There are reports of differences in response to applied auxins among roots from different species (1), among different varieties of the same species (64), or even in roots of different ages of the same species (105). Burström (40) has further pointed out that cells at different stages of development within a single root show different sensitivities to applied growth substances, including the auxins.

The difference in sensitivity of roots of different species is in itself a problem of considerable interest. As early as 1936, Thimann (137) had suggested that the difference in sensitivity to applied auxin of roots of *Avena* and *Pisum* was determined by the level of endogenous auxin and that therefore *Avena* roots, which already contained high quantities of auxin, were much more sensitive to supplied auxin than *Pisum*, in which the endogenous auxin level was suboptimal for normal elongation. This idea was substantiated by direct extraction of these roots for auxin. Åberg (7) suggested a similar mechanism to explain the observed higher sensitivity to applied auxins exhibited by flax roots as compared with wheat roots.

Pilet (105) found a striking difference in sensitivity of roots of *Lens* to applied auxin, dependent upon the age of the seedling root, which, in turn, is correlated with a general increase in endogenous auxin level during root elongation. Maximum stimulation of one-day-old roots by IAA was obtained at 10^{-7} *M* and inhibition began at 10^{-5} *M*. Six-day-old roots were stimulated maximally at 10^{-9} *M* IAA and inhibited by concentrations of 10^{-5} *M* or higher. Twelve-day-old seedling roots of *Lens* showed maximal stimulation of elongation at 10^{-11} *M* IAA with inhibition beginning at 10^{-8} *M* or higher. Pilet has interpreted these differences in sensitivity in terms of suboptimal levels of endogenous auxin in the roots which are augmented by externally

supplied auxin either to the level of stimulation or to that producing inhibition. He has shown by direct extractions of roots of different ages that the endogenous auxin levels increase over the 12-day period.

Street, McGregor & Sussex (135), studying the effect of IAA and IAN on growth of excised tomato roots in nutrient medium, have shown that 50 per cent inhibition of linear increase in main axis growth was produced by *circa* 3×10^{-8} *M* IAA, which was equally effective in reducing lateral root initiation. The decrease in root elongation was attributed to reduced cell enlargement. The nitrile was much less effective in root inhibition, causing 50 per cent inhibition at 3×10^{-5} *M* and affected both cell enlargement and cell multiplication, suggesting that these closely-related auxins affect root growth quite differently. This observation confirms the earlier report by Bentley & Bickle (24) that IAN is much less effective than IAA in causing root inhibition in cress roots, but is about as effective in *Avena* roots. It is possible that the effectiveness of IAN as an auxin depends on the capacity of the root tissue to convert it to IAA, as has been shown by Thimann (141) to occur in the *Avena* coleoptile but not in pea stem sections. Street (131) has further shown that IAA applied at noninhibitory concentrations for root growth, i.e., 5.7×10^{-10} *M*, reduces the survival of the main axis meristem, i.e., leads to early cessation of cellular mutiplication in the meristem. IAN, on the other hand, was shown to have no such effect on survival. Evidence for the deleterious effect of IAA on meristematic activity in the tomato root does not lend support to the concept that IAA itself may be a root growth hormone in tomato roots.

Although there are a number of reports of IAA oxidase activity from tissue sections or breis prepared from roots [Galston & Dalberg (63), Wagenknecht & Burris (145), Åberg & Jönsson (8)], there still remains little concrete evidence as to the physiological role of the oxidase in controlling IAA levels in the root tissues, which could in turn control root elongation. The existence of such a system, however, which is active at cut surfaces especially in destroying auxin in solution (8), may be of importance in planning experimental procedures and in the interpretation of results. Åberg (7) has shown that pea root sections have much higher IAA oxidase activity than wheat root sections. He points out that 2,4-D, which would not be oxidized by the IAA oxidase, was much more active in inhibiting root elongation than IAA itself in the pea root system. The rapid destruction of certain applied auxins such as IAA but not other compounds, such as 2,4-D or NAA, is in itself another source of confusion in the literature. Åberg & Jönsson (8) presented further evidence showing that IAA oxidase may itself vary greatly in activity in root sections taken from different species.

In view of the complexity of the phenomenon of root inhibition by auxins, it is evident that considerable attention must be paid to all the possible factors influencing root elongation. Results obtained with different test objects and under different experimental conditions are likely to appear contradictory, and all due consideration must be given to the possible effects

of external or internal conditions in interpretation of results if erroneous conclusions are to be avoided. The sources of error mentioned above become particularly important when reversal of auxin inhibition of root elongation is studied using auxin antagonists or antiauxins.

In the light of the manifest complexities, the agreement concerning the fact of auxin inhibition of root elongation is perhaps even more striking. Compounds of the auxin type are extremely potent inhibitors of root elongation at concentrations from 100 to 1000 times lower on a molar basis than that of the usual metabolic inhibitors. This fact suggests that the nature of auxin action in root inhibition is distinctive and not to be compared directly to inhibitions such as those produced by respiratory enzyme inhibitors or antimetabolites.

Burström (38) has long maintained that cell elongation starts with auxin-stimulated dissolution of the cell wall which becomes both plastically and elastically extensible. This dissolution or plasticizing of the wall was conceived to be possible because of the predominantly pectic nature of the primary cell wall, with the first phase of cell enlargement occurring passively as a result of water absorption at the expense of wall thickness. In the second phase, the main part of elongation which is inhibited by auxin, Burström believed new cell wall materials were deposited by the intussusception of new cellulose micelles, with the pectic materials forming an intermicellary framework. In recent years Burström (47, 50) has modified this two-phase scheme for cell growth and has extended the experimental basis for his view by his extensive studies of cell growth in roots. He has concluded from osmotic and auxin studies of root elongation (47) that it is the condition of the cell wall which generally limits cell growth.

Burström (47) interprets the auxin effect as acting to dissolve the cell wall, i.e., an acceleration of the first phase of cell enlargement. On the other hand, auxin acts on the second phase of cell enlargement as well by shortening its duration, thereby retarding cell enlargement which results from accelerated hardening of the wall. Recently, Burström (50) has made the further suggestion that cessation of root cell elongation may, in fact, be caused by a decrease in auxin concentration during cell elongation to a level which no longer allows plastic extension of the wall, i.e., that fixation of the wall results when auxin is no longer present in adequate concentration. It is not clear from his work whether auxin is conceived to act directly in affecting the properties of the cell wall or indirectly by an effect on the metabolism of the cell. Calcium also has important effects on root growth according to Burström (46, 49) because of its effect on the root cell wall where it is essential for wall elasticity in the initial phase of enlargement and plays some undetermined role in cell wall growth by intussusception in the final phase.

Auxin stimulation.—Still more remarkable is the frequently reported observation that auxins in very low dilution, i.e., *circa* 10^{-11} *M*, cause a significant stimulation of root elongation. This effect, even more than that

of inhibition, is subject to the conditions of the experimental test. Åberg (7) recently questioned the reality of such a stimulation by IAA in *Lepidium* and reviewed some of the cases reported in the past.

The reality of a specific stimulation of root elongation by low concentrations of auxins is a matter of considerable theoretical importance, especially in the interpretation of the possible role of naturally-occurring auxins in controlling root elongation. As has been pointed out by Thimann (140), growth stimulation effects are more specific than growth inhibitions. It is therefore of interest to review the recent reports of the specific stimulation of root elongation by auxins and related compounds. The observation of auxin stimulation of root elongation was made first in 1936 by a number of workers (see 140) and has turned up repeatedly ever since that time.

More recently Moewus (96) reported a 10 to 15 per cent stimulation of root elongation in *Lepidium* roots by IAA at concentrations of about 5.7×10^{-10} to 5.7×10^{-12} *M*. Clauss (53) was able to obtain similar stimulations with the same test, but Müller (100) could not confirm these observations. Moewus & Moewus (97) reported that sensitivity depended upon the natural auxin content of the cress roots. They found young roots (5 to 6 mm. long) contain twice the concentration of auxin as long roots (20 to 25 mm. long) which were in a phase of decreasing growth-rate. Only during this last phase did the roots respond to added low levels of auxin. They also claimed that seedling roots from different seed lots varied considerably in auxin content and therefore in their responses to added auxin.

Pohl (110) reported a consistent stimulation of cress root elongation of about 10 to 15 per cent by IAA at 5.7×10^{-11} *M* in a test similar to that used by Moewus. It is this stimulatory effect which he considers to be distinct from the inhibitory effects of auxin on root elongation. Pohl & Ochs (113) and Ochs (102) have shown inhibition of auxin-stimulated root elongation by DNP and KCN, but not by iodoacetic acid. They reported up to 14 per cent stimulation over control growth by IAA at 5.7×10^{-13} *M*. They also reported 23 per cent stimulation over the control growth by 4×10^{-14} *M* 2,4-D and 15 per cent stimulation by 2×10^{-12} *M* triiodobenzoic acid! Ashby (14) established statistically significant stimulation of *Artemisia* root elongation (14 per cent) by IAA at 5×10^{-10} *M* and for indoleacetaldehyde (10 per cent) at 5×10^{-12} *M*. In all of these cases, intact seedling roots were studied.

Audus & Garrard (19), studying the effects of auxin on root section elongation, found a 30 per cent increase in section length caused by 10^{-11} *M* IAA. This stimulation was independent of sucrose supply to the sections and persisted for experimental periods up to 48 hr. Audus & Thresh (21), using increase in fresh weight of 2 mm. pea root sections as a measure of growth, reported significant stimulations by IAA concentrations of 10^{-11} *M* (18 per cent) and 10^{-10} *M*. No stimulation of this sort was observed with 2,4-D. Inhibition was still obtained at 10^{-11} *M* (6 per cent) and 10^{-10} *M* (8.8 per cent).

It is interesting that Kandler (78) and Kandler & Vieregg (81) were able

to show a slight stimulation (*circa* 20 per cent) of root elongation of isolated corn roots in culture by additions of 10^{-9} *M* IAA, although on a dry weight basis treated roots showed lower dry weight increases than control roots or roots treated with inhibitory concentrations of IAA. Kandler (78) was unable to demonstrate a similar stimulation of root elongation with 2,4-D or NAA at low concentrations. If the above data of Pohl & Ochs (113) concerning 2,4-D stimulation are significant, it would seem that failure to demonstrate 2,4-D stimulation may be attributable to insufficient dilution. Jagendorf (75) reported an initial marked stimulation of elongation of isolated cabbage roots in culture in the presence of 10^{-8} to 10^{-9} *M* IAA or 10^{-9} *M* *p*-chlorophenoxyacetic acid.

The results of Pilet (105, 107) on the sensitivity of roots of *Lens* to added auxin have already been discussed. He also found striking stimulation of root elongation at low auxin concentrations, depending upon the age of the root, the illumination which the roots received, and other conditions affecting the internal auxin concentration of the root. Pilet & Pfister (108) also reported that low concentrations of IAA stimulate root elongation in *Iris* while causing inhibition at high concentrations with sensitivity dependent upon the age of the root and its exposure to light.

Baldovinos (22) likewise reported the stimulation of root elongation in excised segments of *Zea* roots cultured in nutrient medium with added auxin. He found that 10^{-9} *M* IAA produced the greatest stimulation, resulting in a tenfold increase in length of the apical 2 mm. segment, a fourfold increase of the 2 to 4 mm. segments, and a doubling of the length of the 4 to 6 mm. segment in a 24 hr. period. Roberts & Street (118) recently made the interesting discovery that isolated roots of rye, which had previously proved difficult to maintain in culture, show marked stimulation of growth in culture, particularly of lateral branches, when supplied with 5.7×10^{-9} to 5.7×10^{-8} *M* IAA. The closely related indoleacetonitrile causes equally stimulatory effects at about the same concentrations.

Despite the questions raised concerning auxin stimulation of root elongation by Åberg (7), there seems to be little doubt that under many different conditions and in different plant species real stimulation may occur. It should be pointed out, however, that stimulatory effects on root elongation are not restricted to auxins. Recent reports suggest that many nonauxin compounds may produce statistically significant stimulation of root elongation. Cooil (54) found that sodium and potassium salts of fumarate, pyruvate, acetate, and malonate at 10^{-3} *M* all produced stimulation of root elongation. This result was interpreted as a possible utilization of the organic acids in metabolism, except for the stimulatory effect of malonate which remained unexplained. Stimulation by other organic compounds may be subject to interpretation in terms of direct utilization as either metabolites or growth factors. Thus, Fries (62) reported an elaborate analysis of the effects of amino acids, purines, pyrimidines, and other organic compounds on root elongation in cotyledonless pea seedlings. He found that root elonga-

tion of decotyledonized pea seedlings could be largely restored to that of intact seedlings by providing the roots with a synthetic nutrient medium containing the organic supplements arginine, glycine, and adenine. Ornithine or citrulline could replace arginine.

Allen & Skoog (10) have shown that certain aromatic derivatives of succinimide stimulate root elongation in wheat and radish seedlings. It was suggested that their action may be attributable to effects on permeability or by interfering in some way with the auxin metabolism of the roots.

Deysson (56) made the interesting observation that adenine applied to pea roots at 10^{-5} *M* stimulates root elongation by 35 per cent, although at concentrations of 5×10^{-3} *M*, adenine inhibits root elongation and causes abnormalities in cell divisions. Adenylic acid and adenosine, while inhibitory to root elongation at concentrations of 10^{-4} *M*, show no stimulatory effect at lower concentrations [Deysson (57, 58)]. More difficult to interpret are the reports of stimulation of root elongation by low concentrations of metabolic inhibitors. Thus Kandler (77) observed a stimulation of corn root elongation *in vitro* in the presence of 1.7×10^{-6} *M* DNP, a stimulation which persisted over a ten-day period and then disappeared. Kandler (78) also observed a slight but significant stimulation of root elongation in cultivated *Zea* roots in the presence of 10^{-4} *M* sodium fluoride.

There are earlier reports of nonspecific root stimulation by other compounds which are difficult to understand or interpret in any general mechanism. Lane (86) showed that dilute (5×10^{-6} *M*) HCl stimulated root elongation in *Avena* seedlings. Similarly, stimulations of root elongation by dilute $CuSO_4$, by certain surface active agents, and other miscellaneous reagents have been reported [see Ashby (14)].

In surveying the effects of auxins and other compounds on stimulation of root elongation it is again strikingly apparent that the auxins are, on a molar basis, by far the most effective agents causing this response, a fact which points to the distinctive nature of their activities in root tissues.

Auxin Antagonisms and Root Elongation

In recent years among the most interesting compounds discovered which affect root elongation are the so-called auxin antagonists or antiauxins. There is at present no agreement upon a terminology to be applied to substances which antagonize auxin activity. Antiauxins have been defined [Overbeek *et al.* (104)] as compounds which inhibit competitively the action of auxins. McRae & Bonner (94) have stated that the only rigorous method of demonstrating auxin antagonism is by application of the Lineweaver and Burk kinetic analysis of competitive inhibition. To date, so far as the author is aware, such analysis has not been applied to root elongation phenomena. Audus (18) discussed the difficulties, both practical and theoretical, of adhering rigorously to such a definition. Burström (51) also discussed the unsatisfactory nature of this definition. It has been suggested by some authors [e.g., Audus & Shipton (20] that auxin antagonism is, in fact, most

satisfactorily demonstrated in the relief of auxin inhibition of root elongation. Hansen (71) has gone so far as to suggest that compounds which stimulate root cell elongation (previously called antiauxins) should be termed root auxins as distinct from shoot auxins. Gordon (68) suggested that the broader term auxin antagonist be applied to compounds which inhibit auxin action, but whose effects are to some degree reversed by auxin. The more closely prescribed qualification of competitive or non-competitive inhibition could than be applied when appropriate.

There seems little doubt but that much of the present confusion in the literature regarding the nature of auxin antagonisms in root growth has developed from loose usage of terms applied to ill-defined physiological systems. In studies of the control of root elongation by auxins and auxin antagonists, the following points must be taken into account. In the first place, there are the established facts that externally applied auxins at very low concentrations stimulate and at higher concentrations inhibit root elongation. In the second place, there is the theory, widely held but not rigorously proven, that endogenous auxin levels determine the rate of root elongation. These two points have been brought together in the past in explaining the response, either positive or negative, of roots to externally supplied auxins. When turning to the study of the effects of auxin antagonists on root elongation, it is essential to distinguish between these factors. Three categories of active compounds must be recognized. (*a*) Compounds related to auxin in chemical structure which in themselves show little or no effect on root elongation at a given concentration but which effectively prevent the inhibition caused by added auxin may be called auxin antagonists and may be qualified as competitive antagonists when this fact is experimentally established. (*b*) Compounds which by themselves markedly stimulate root elongation, but which also prevent inhibition of root elongation by externally supplied auxin, may also be classified as auxin antagonists. (*c*) Compounds which by themselves cause stimulation of root elongation but which have no effect on auxin-induced inhibition of root elongation should not be characterized as auxin antagonists until such time as it has experimentally demonstrated that their activity depends upon inhibition of root elongation under the control of endogenous auxin.

The difficulty in the use of the term antiauxin as defined above lies in the further definition of competitive inhibition and its experimental demonstration. In the restricted sense used by McRae & Bonner (94) no demonstration of antiauxin activity has been forthcoming from studies of root elongation. For the present discussion, the term antiauxin will not be used; rather the term auxin antagonist will be used in the general sense proposed by Gordon (68) as qualified above.

The concept of auxin antagonism and antiauxin action has been discussed extensively in the recent literature (1, 4, 18, 29, 71, 88, 94, 99, 103, 128). It is not the present purpose to discuss the validity of the antiauxin concept, the structural requirements for antiauxin activity, the experimental

determination of antiauxin action either in shoot or root tissues, or the mechanism of antiauxin action. Rather, it seems appropriate to summarize the present status of our knowledge of auxin physiology in root growth phenomena as a result of the extensive recent work on the effects of auxin antagonists on root elongation.

As pointed out by Bonner & Bandurski (29), auxin antagonists are substances related in chemical structure to the auxins. When acting competitively, their effectiveness depends on the inhibitor/auxin ratio, and their action may thus be conceived as comparable to known antimetabolite-metabolite relations. Thus, when considering auxin antagonisms in growth phenomena, one must be concerned not only with the quantity of externally supplied auxin but also with the endogenous auxin of a tissue which, according to theory, should also be inhibited in its action. It is this last aspect of the auxin antagonism studies which bears close scrutiny as a possible key to information concerning the role of natural auxins in root elongation. There has been interest also in the effects of auxin antagonists on root geotropism with the thought that understanding of the mechanism of this response might also be clarified [cf. Netien & Canillot (101), Pilet & Wurgler (109), Rufelt (121), Hansen (71)].

McRae & Bonner (94) listed some 25 different compounds which have been reported as active auxin antagonists in root elongation. These compounds include, among others, the isobutyric acid derivatives studied by Burström (41 to 44), Åberg (4), and Kandler & Eberle (79); α-(1-naphthylmethyl-sulfide) propionic acid (NMSP) and related compounds studied by Åberg (1, 2, 4) and Street (130, 131); the phenoxyacetic acid derivatives whose effects on root elongation have been reported by Åberg (4) and Audus & Shipton (20), and other compounds structurally similar to known auxins.

A number of compounds reported to be auxin antagonists in *Avena* coleoptile elongation or shoot growth have been reported ineffective in preventing auxin inhibition of root elongation. Thus γ-phenylbutyric acid has been found by Åberg (4) to exert only an inhibitory effect on root elongation in *Linum* seedlings. Similarly, coumarin has been shown repeatedly to produce only inhibition of root elongation which, when applied together with auxin, is additive in its effect [Moewus (96), Pollock *et al.*, (114)]. Audus & Shipton (20) have given evidence that 2,4-dichloranisole shows only a very slight antagonism in pea root section growth.

Triiodobenzoic acid (TIBA), which was initially reported (1) only to inhibit root elongation of flax seedlings in pure solution and to increase the inhibition of applied auxin, was subsequently found by Åberg (5) to behave in a complex fashion in its interaction with applied auxin. With increasing concentration it acts first as an auxin synergist enhancing root inhibition, then as auxin antagonist counteracting auxin inhibition, and finally as a toxic agent. This interpretation of TIBA effects in root growth has been questioned by Audus (18). Gorter (69) has made the interesting, if presently

inexplicable, observation that TIBA inhibits root hair elongation in roots of *Lepidium* apparently without interfering with the formation of cell wall materials usually associated with root hair elongation.

Pohl & Ochs (113), on the other hand, reported that TIBA acts in every respect as an auxin when tested with *Lepidium* roots, i.e., stimulating root elongation at low concentration and inhibiting at higher concentrations. A similar view has been taken by Kandler & Fink (80) who found TIBA at 10^{-7} *M* to stimulate elongation of isolated *Zea* roots grown in culture, but at higher concentrations to inhibit root elongation and to produce increased root diameters comparable to those caused by IAA. They conclude that TIBA behaves as an auxin in root growth and clearly not as an auxin antagonist.

Schopfer *et al.* (127) presented data showing that maleic hydrazide by itself and in the presence of stimulatory concentrations of IAA inhibits root elongation of isolated pea roots in culture, but relieves slightly the inhibiting effect of higher auxin concentrations when it is not in itself at inhibitory levels. Maleic hydrazide has been found to have little effect on root elongation in *Linum* at low concentrations and acts only in an inhibitory fashion at concentrations of 10^{-4} *M* or higher [Åberg (5)]. In the presence of inhibitory concentrations of IAA (but not 2,4-D) Åberg claimed maleic hydrazide shows a weak auxin antagonism. In the light of reports [e.g., Greulach & Atchison (70)] of a particularly potent effect of maleic hydrazide on mitotic activity of root tips, the interpretation of this action as auxin antagonist is perhaps to be questioned. Pilet (106) found that maleic hydrazide inhibits root elongation in *Lens* whether applied to young or to old seedling roots which are believed to be quite different in endogenous auxin. Insofar as root elongation is concerned, there is little clear-cut evidence that maleic hydrazide should be termed an auxin antagonist.

The auxin antagonists reported from root elongation studies behave in different ways. One group of compounds, (*a*), appears to have little effect on root elongation in pure solution, but they specifically counteract the effect of added auxin. The second group (*b*) includes compounds which are in themselves effective in stimulating root elongation and in the presence of added auxin counteract its activity. Concentration is of great importance in these effects and usually at sufficiently high concentration the compounds become inhibitory. The distinction between compounds (*a*) and (*b*) is often difficult to make experimentally. Audus & Shipton (20) and Audus (18) have accepted as "firmly established" auxin antagonists only those in which the antagonist removes auxin-induced growth inhibition at concentrations where it has in itself substantially no effect. Such antagonisms have been shown for α-(1-naphthyl-methyl-sulfide) propionic acid (NMSP), α-*p*-chlorophenoxyisobutyric acid (PCIB) (41, 42), (-)α-(2-naphthoxy)-propionic acid (3), phenoxyacetic acid (4), α-phenoxyisobutyric acid (4, 43) and in addition a number of substituted phenoxyacetic acids and phenoxy-

propionic acids (6). Many of these compounds are far more effective in relieving root inhibition produced by 2,4-D than that caused by IAA, a fact that is not clearly understood. Furthermore, many of the antagonists show their greatest effects when applied as pure compounds. Stimulations of root elongation by 100 per cent or more have been reported for certain isobutyric acid derivatives (43). Thus, for example, (41) PCIB at 3×10^{-6} *M* when applied to wheat roots produces 75 per cent stimulation of root elongation over that of the control, wheras IAA at 10^{-6} *M* causes 70 per cent inhibition of root elongation. When applied together at these concentrations, the antagonist decreases the auxin inhibition by about 10 per cent. PCIB thus is typical of group (*b*) compounds. In contrast, Åberg (1) has found NMSP at 2×10^{-5} *M* causes essentially no effect on root elongation in *Linum* seedlings. When applied together with 10^{-7} *M* 2,4-D, the marked inhibition (75 per cent) which is caused by the auxin alone almost completely disappears. NMSP belongs in group (*a*). In these various effects on root elongation, there has been observed a marked difference in behavior of different species of plants, so that generalizations cannot be made from observations on only one plant system. Compounds in group (*a*) for one plant may belong in group (*b*) for another plant species.

There is at the present time no clear understanding of the mechanism of action of the auxin antagonists on root elongation. Many interpretations have been proposed and none has been acceptable in all its aspects. Åberg (1 to 8) has adhered to the concept that the auxin antagonists which he has reported are acting as true competitive inhibitors of auxin. He maintains that, in general, endogenous auxin levels are supraoptimal for maximum root elongation and added antagonists bring the endogenous auxin levels to stimulatory concentrations. He has elaborated upon the relations among weak and strong auxins and auxin antagonists in terms of their differential affinities and activities (4), which are specific properties of the compounds derived from their chemical structures. In defending his position, Åberg has pointed out the questionable nature of the evidence for stimulation of root growth by auxins and has emphasized the significant differences in antagonist effects on roots inhibited with IAA, a natural compound, and 2,4-D, the synthetic auxin. He implies that IAA may be the natural root growth hormone which is antagonized by the added compounds.

Audus (18) discussed the dilemma which the recent evidence of auxin antagonism in root elongation poses with respect to the interpretation of the role of auxin in root growth. If one accepts as real the stimulatory effect of added auxin on root elongation in many roots, it becomes difficult to explain the stimulation of elongation in the same roots by auxin antagonists in simple terms of competitive inhibition of endogenous auxin. On these and other grounds, Audus & Shipton (20) and Audus (18) question whether the natural growth substance in roots is a compound of the IAA-type at all. They suggest that root elongation may be controlled by a natural inhibitor

which maintains root elongation below its maximum and that the auxin-like compounds counteract this natural inhibition, in accordance with their chemical affinities and activities. They point out further that an antagonist may affect growth at more than one site in the sequence of events leading to elongation and that the multiplicity of antagonist actions must be carefully analyzed and taken into account.

Street (131, 132), from studies of the effect of IAA and NMSP on growth and survival of excised tomato roots in culture, concluded that a hormone which accumulates in the apical region is responsible for the inhibition of elongation and ultimate cessation of cell multiplication. The inhibitor, believed to be auxin-like in structure but not IAA, is antagonized in its action by NMSP and certain other auxin antagonists. The proposed inhibitor would conform in some respects at least to that suggested by Audus (18).

Galston & Dahlberg (63) suggested that auxin antagonists such as PCIB are effective inducers of IAA oxidase activity and thus interact in the control of root elongation by this indirect effect on endogenous auxin levels. This view has been criticized by Åberg (7).

Considerable skepticism over a simple interpretation of auxin antagonism in root elongation has been expressed by Skoog (128). He discussed the difficulties of interpretation of the optimum root growth curve response in terms of endogenous auxin levels and the effects of added auxins or auxin antagonists. Instead of a system in which inhibition depends upon competition for a single site, Skoog visualizes a more complex situation in which simultaneous interaction of two substances at several different sites may occur. Thus stimulation and inhibition of growth by an auxin antagonist or inhibition and synergism of added auxin by an antagonist could be explained on the basis of differential affinities of the two substances for sites in different complexes, only one of which participates in the growth reaction.

Bitancourt (25) in discussing this problem pointed to the multiplicity of actions produced by auxins and suggests that compounds which effectively antagonize one auxin action may have no effect or may actually stimulate another auxin action. In roots, both of these actions may be a part of the over-all process leading to root elongation.

Burström (43) has, in fact, maintained that in cell enlargement during root elongation, auxin has a dual action—first a positive effect, and then a negative effect. He has interpreted auxin antagonist action in terms of differential antagonism of these two separate auxin effects so that the resultant elongation is the integration of these interactions. The "ideal antagonism" is an antagonism of the negative auxin action so that stimulated root elongation results. By such an ingenious theory, one can readily account for all degrees of stimulation or inhibition by adjustment of the two phases and their contribution to the whole process in the presence of external reagents. Unfortunately, as Burström himself admits, the theory is not readily tested experimentally. The interaction between auxin and antagonist during the

first phase is difficult to analyze experimentally because of the relatively small positive effects of auxin (41). Furthermore, the demonstration of experimental separation of phase one and phase two has not been easy, and therefore Burström's criteria of these phases have not been generally applied in other laboratories in the study of root growth. If auxin affects two separate processes during cell elongation, it is clearly desirable that these two processes be characterized more specifically so that they may be subjected to analyses leading to clarification of auxin antagonist effects.

It is apparent from the preceding discussions on auxin inhibition and stimulation of root elongation and on auxin antagonism in root elongation that there exists an urgent need for clear indisputable evidence for or against the direct involvement of endogenous auxin in the control of root elongation. Only after establishment of the role of endogenous auxin in root elongation will it be possible to discuss realistically the mechanism of action of supposed auxin antagonists and other inhibitors of root elongation.

XUM

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MECHANISMS OF CARBOHYDRATE BREAKDOWN IN PLANTS[1,2]

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The objective of this review, in deference to its title, should be to sum up the more important recent developments of carbohydrate metabolism in plants which have a bearing on the possible routes of breakdown. The term "breakdown" is certainly unequivocal; the conversion of, e.g., glucose to carbon dioxide and water can be nothing other than "breakdown." However the adventures of the glucose carbon atoms on their journey to the ultimate of all ends, carbon dioxide, are for considerable distances indistinguishable from those of carbon atoms which are destined for posts as tissue constituents. In short, then, it is difficult to avoid a serious infiltration here of matters dealing with "carbohydrate transformations." In regarding the intermediates between carbohydrate and carbon dioxide, the concept of "carbohydrate breakdown" is largely a point of view.

A major part of this review will deal with the emergence of a new pathway of glucose dissimilation as an important recent development in plant physiology. The component reactions of this sequence and their possible significance will be discussed after a brief review of the status of our knowledge of the classical EMP (Embden-Meyerhof-Parnas) route of glucose breakdown. Pertinent recent developments in the area of simple carbohydrate transformations in plants will then be considered.

Experience has amply demonstrated that biochemical patterns tend to be similar in many forms of life. Therefore, allusions to recent developments outside the domain of higher plants will be included here in the belief that this information will be of value in future studies in this field.

The Embden-Meyerhof-Parnas Pathway

The realization by Buchner that a yeast extract could convert glucose to carbon dioxide and ethanol was, as is well known, the beginning of our understanding of carbohydrate metabolism. The knowledge that phosphate was required, that intermediates accumulated, and that various co-factors were

[1] The survey of the literature pertaining to this review was concluded in November, 1955.

[2] The following abbreviations will be used in this chapter: ADP for adenosinediphosphate; ATP for adenosinetriphosphate; DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; EMP for Embden-Meyerhof-Parnas pathway; P/O for ratio of moles of inorganic phosphate taken into organic combination (ATP) to the atoms O consumed; TPN for triphosphopyridine nucleotide; TPNH for reduced triphosphopyridine nucleotide; UDPG for uridine diphosphoglucose; UTP for uridine triphosphate.

needed for the fermentation made it clear that this was no simple one-step conversion. This connected series of reactions, the EMP pathway, is so well known even outside the discipline of biochemistry that one hesitates to dwell on it in any detail. In the present review only a brief outline of the EMP pathway will be given, and comment will be restricted mainly to recent information from plants. Recent reviews of this subject as it applies to higher plants have been published by Stumpf (1, 2). A schematic outline of the pathway is shown:

$$\text{Starch} + PO_4 \rightarrow \text{glucose-1-}PO_4$$

$$\text{Glucose} \xrightarrow{ATP} \underset{\downarrow}{\text{glucose-6-}PO_4} \rightarrow \text{fructose-6-}PO_4 \xrightarrow{ATP} \text{fructose-1,6-di}PO_4 \rightarrow$$

$$(\text{dihydroxyacetone-}PO_4 \leftrightarrow \text{glyceraldehyde-3-}PO_4) \xrightarrow[PO_4]{DPN} \text{1,3 diphosphoglycerate} \xrightarrow{ADP}$$

$$\text{3-phosphoglycerate} \rightarrow \text{2-phosphoglycerate} \rightarrow \text{phosphoenol pyruvate} \xrightarrow{ADP}$$

$$\text{pyruvate} \xrightarrow{-CO_2} \text{acetaldehyde} \xrightarrow{DPNH} \text{ethanol}$$

The EMP pathway, historically, was developed to account for the conversion of glucose and starch to ethanol and carbon dioxide. However, in its general application the formation of ethanol and acetaldehyde may be considered as a special case, and one may regard the pathway as terminating at pyruvic acid. The further conversion of pyruvic acid to carbon dioxide, which completes the breakdown of glucose, requires some provision for its oxidation. In plants, that provision is probably met by the Krebs carboxylic acid cycle which will be alluded to briefly below. It should be further noted that in the classical glucose to ethanol transformation DPN is regenerated from its reduced form by the act of acetaldehyde reduction. In plants, too, this appears to be the general pattern, although in a few cases, e. g., potato, some of the pyruvate may be reduced to lactate [Barron *et al.* (3), Barker & & El Saifi (4)]. Thus the EMP pathway as shown is self-sufficient in that it requires no external electron acceptor such as oxygen. For this reason the EMP pathway is sometimes referred to as the anaerobic pathway or, synonymously, the fermentative pathway. These names should be honored for their historical value, but it should be recognized that the series of reactions will proceed equally well whether the pyridine nucleotide is reoxidized (ultimately) by oxygen or by another substrate. With the isolation of phosphoglyceryl kinase from peas by Axelrod & Bandurski (5) and of hexokinase by Millerd *et al.* (6) and by Kotelnikova (7) the list of EMP enzymes isolated from higher plants was completed. For the skeptic, of course, there remain countless species which have not yet been examined. However, in view of the numerous instances in which plants and extracts or powders derived from them have been shown to bring about alcoholic fermentation, it is safe to say that most observers consider it highly probable that these enzymes occur in all higher plants.

Triosephosphate dehydrogenase.—Nevertheless, even among those tissues already examined, there are some variations on the general theme and more may be anticipated. For example, three different enzymes which can accomplish the dehydrogenation of glyceraldehydephosphate are present in higher plants. The first is the conventional dehydrogenase which can function only in conjunction with DPN. Tewfik & Stumpf (8) had earlier reported its absence from pea leaf tissue in contrast to its presence in the ungerminated pea. Gibbs (9) showed that appreciable amount of this enzyme could be extracted from the leaf if care were taken to avoid the oxidation of the sensitive sulfhydryl groups in the enzyme. Simultaneously he demonstrated that a TPN requiring analog of this enzyme was also present. The occurrence of this latter enzyme was also shown by Arnon (10) and by Axelrod & Bandurski (11). Gibbs (12) succeeded in purifying this second enzyme from pea leaves and found it completely specific for TPN. Like the conventional DPN enzyme it could be accelerated by arsenate. Still a third triosephosphate dehydrogenase, which required TPN but whose activity was independent of the coupled phosphorylation of ADP (and therefore was not stimulated by arsenate), was discovered in spinach leaf extracts by Axelrod *et al.* (13). What appears to be a similar enzyme has now been purified from sugar beet leaves by Arnon *et al.* (14). These authors demonstrated that the three types of triosephosphate dehydrogenase were present in the original extracts. Hageman & Arnon (15) described the isolation and considerable purification of the DPN-requiring enzyme from pea seeds and followed in some detail the changes which occur in the levels of the enzymes requiring DPN and TPN during the life cycle of the pea plant (16).

Hexokinase.—Since the last review on glycolytic enzymes in this series [Stumpf (1)] more detail has appeared on plant hexokinase. Saltman (17) reported the presence and some of the properties of this enzyme in a number of plants. Kotelnikova (7) has shown it to be present in the potato. In contrast to the findings of Millerd *et al.* (6) which indicated that the enzyme is particulate, Saltman found at least some of the enzyme to be dissociable from the particulate matter. The fact that the enzyme is particulate, at least in part, provides an exception to the rule that the enzymes of the EMP pathway or the pentose phosphate pathway (see below) are soluble.

Enzymes of the Pentose Phosphate Pathway

The last few years have seen a renewed interest in the fate of the products of the enzymatic oxidation of glucose-6-phosphate which was first described from animal tissues some twenty years ago [Warburg *et al.* (18), Dickens (19, 20)]. The result of this has been the recognition that the pentose phosphate produced from the oxidative decarboxylation of 6-phosphogluconate undergoes a complex sequence of reactions involving isomerizations, the transfer of C-2 and C-3 pieces, the synthesis of a 7-carbon sugar as an intermediate, and, eventually, the resynthesis of hexose phosphate. This reaction sequence has been variously referred to as the direct oxidation

pathway, the Warburg-Lipmann-Dickens pathway, the oxidative glycolysis pathway, the hexose monophosphate shunt, and the phosphogluconate shunt. Following Horecker & Mehler (21) the name "pentose phosphate pathway" is used in this review. A great deal of progress has already been made in breaking down this complex system into its component reactions and in isolating the enzymes concerned. Recent reviewers in the field [Dickens (22), Racker (23), Horecker & Mehler (21)] have been able to construct tentative reaction sequences which are solidly based and which account for most of the observed reactions. Such a sequence is shown in Figure 1.

Investigations on enzymes from plants have contributed materially in making possible the rather dramatic delineation of the reaction sequence in the last few years. Some of the key enzymes were purified first from plant sources. Also, experiments on the pentose phosphate to hexose phosphate conversion in extracts from higher plants have led to some of the deductions summarized in Figure 1. In the following paragraphs the development of our knowledge about these processes in plants is summarized although some of the major points have already been considered in recent reviews mentioned above. It will be seen that there is evidence from experiments on plant extracts for each of the reactions shown in Figure 1. A convincing argument can be presented for the potential operation of the cycle *in vivo*. Before discussing the various individual reactions it is of interest to note that Gibbs (24) has recently shown that various intermediates of the sequence shown in Figure 1 can strikingly promote the uptake of oxygen in extracts from pea leaves when TPN is supplied. Oxidation of glucose-6-phosphate, 6-phosphogluconate, and ribose-5-phosphate (but not of the phosphate-free compounds) was observed, and the O_2 uptake was substantially resistant to 0.01 *M* cyanide.

Glucose-6-phosphate dehydrogenase.—The discovery in higher plants of the enzymes of what are now known to constitute the pentose phosphate pathway may be said to have begun with the report by Wagner-Jauregg & Rauen (25) that glucose-6-phosphate dehydrogenase (Zwischenferment) is present in a number of seeds. With the renewed interest shown in these reactions in the early 1950's came several reports of the occurrence of this enzyme in plant materials generally. Conn & Vennesland (26) showed that extracts of wheat germ could reduce TPN when glucose-6-phosphate was provided. In a subsequent report by Anderson *et al.* (27) the enzyme was shown to be present in a variety of plant parts among which the cucumber fruit was outstanding. In most of the extracts there was an active glutathione reductase which reduced added glutathione at the expense of the TPNH produced by the action of glucose-6-phosphate dehydrogenase. The reaction could thus be followed conveniently by titrating the reduced glutathione which accumulated during a reaction in which the TPN was acting catalytically. Gibbs (9) showed that preparations from the pea plant contained a similar enzyme which was also TPN-specific. The enzyme was

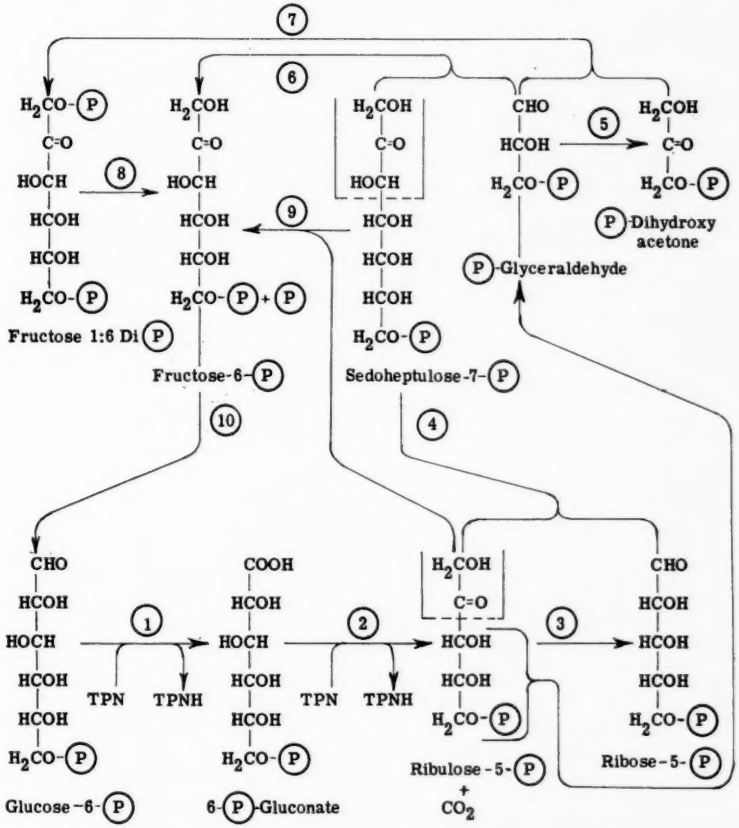


FIG. 1. Reactions of the pentose phosphate cycle, The individual reactions are catalyzed by the following enzymes: 1. Glucose-6-phosphate dehydrogenase; 2. 6-Phosphogluconate dehydrogenase; 3. Phosphoriboisomelase; 4. Transketolase; 5. Triosephosphate isomerase; 6. Transaldolase; 7. Aldolase; 8. Phosphatase; 9. Transketolase; 10. Phosphohexose isomerase.

shown to be present in acetone powders prepared from spinach and pea leaves by Axelrod & Bandurski (11) and Axelrod *et al.* (13). The enzyme has not been highly purified from plant material. It was shown that 6-phosphoglucono-δ-lactone was formed as an intermediate in the reaction catalyzed by the yeast enzyme [Cori & Lipmann (28)]. Brodie & Lipmann (29) recently described an enzyme from *Azotobacter vinelandii* which hydrolyzes

this intermediate to the acid. No investigation of the occurrence of the lactone-hydrolyzing enzyme in plants has yet been reported.

6-phosphogluconate dehydrogenase.—Conn & Vennesland (26) studying glucose-6-phosphate dehydrogenase in wheat germ, found that CO_2 was produced and suggested that 6-phosphogluconate dehydrogenase might be present. Subsequently Barnett *et al.* (30) in the same laboratory, and Axelrod & Bandurski (11) established by direct tests that this enzyme was present in a number of plants. The enzyme could be demonstrated by direct observation of TPN reduction, by glutathione reduction when coupled to the TPNH-requiring glutathione reductase, and by measurement of the pentose and CO_2 liberated. Axelrod & Bandurski found that generally in plant extracts the 6-phosphogluconate dehydrogenase activity greatly exceeded the glucose-6-phosphate dehydrogenase activity so that added glucose-6-phosphate was converted to pentose phosphate without any accumulation of 6-phosphogluconic acid. They detected ribose-5-phosphate by paper chromatography among the reaction products.

The fate of pentose phosphate.—The subsequent reactions of the products of 6-phosphogluconate oxidation were studied in some detail by Axelrod *et al.* (13). A quantitative study of the reaction products showed at first that one mole of sedoheptulose and one mole of triosephosphate were formed from each two moles or ribose-5-phosphate. In short periods of time pentose phosphate as determined in the orcinol test disappeared more rapidly than the formation of heptulose warranted. After much longer periods heptulose declined while hexose phosphates appeared. The hexose and heptulose phosphates were determined by the application of the cysteine-sulfuric acid hexose test of Dische *et al.* (31). Ribulose was found in the early digestion mixtures. All of the components existed as phosphate esters. These workers also found the enzyme preparation to contain a triosephosphate dehydrogenase which was specific for TPN and which did not require phosphate (see above). Barnett *et al.* (30) obtained qualitative tests for a ketoheptose when ribose-5-phosphate was incubated with parsely extracts. They also observed a reduction of TPN with ribose-5-phosphate, presumably attributable to the action of a TPN-triosephosphate dehydrogenase on the triosephosphate formed in the pentose phosphate breakdown.

Transketolase.—The elucidation of the transketolase reaction and its significance in pentose phosphate degradation in preparations of animal origin [Horecker & Smyrniotis (32), Horecker (33)] was quickly followed by the isolation of a highly purified enzyme from spinach leaves by Horecker, Smyrniotis & Klenow (34). With such a preparation they were able to demonstrate the need for thiamine pyrophosphate as a co-factor in the reaction and the appearance of sedoheptulose-7-phosphate (which was later isolated as the Ba salt and conclusively identified) and glyceraldehyde phosphate in stoichiometric amounts as products when ribose-5-phosphate was the initial substrate. Results obtained by these authors with the spinach enzyme and by Racker *et al.* (35) with crystalline transketolase from yeast

showed that the activity is not restricted to pentose phosphate. For the reaction to proceed the following components are required: (*a*) a donor of a 2-carbon piece among which may be listed ribulose-5-phosphate, L-erythulose, sedoheptulose-7-phosphate, and hydroxypyruvic acid; (*b*) transketolase enzyme with thiamin pyrophosphate and Mg^{++}; (*c*) an acceptor aldehyde such as ribose-5-phosphate, D or L or glyceraldehyde phosphate, glycolaldehyde, or erythrose-4-phosphate.

The fact that the reaction will proceed only in the presence of the acceptor component was the basis for the suggestion by Racker *et al.* (35) that an intermediate "active glycolaldehyde" (complexed in some way with the enzyme) was formed as a result of the initial transfer of the 2-carbon fragment. The breakdown of this active glycolaldehyde required the presence of the acceptor aldehyde. Such an explanation would of course account for the fact that no 2-carbon fragment such as glycolaldehyde could be detected as a free intermediate during the formation of sedoheptulose from pentose phosphate in plant extracts [Axelrod *et al.* (13)]. A recent and significant development has been the discovery by Stumpf (36) that D-xylulose-5-phosphate reacts with transketolase at the same rate as D-ribulose-5-phosphate.

Ribose-5-phosphate—ribulose-5-phosphate isomerase.—Of the various possible reactions catalyzed by transketolase only those involving pentose phosphate appear to be of importance here. In plant extracts the provision of ribulose-5-phosphate or ribose-5-phosphate alone leads to sedoheptulose formation, the requirements for a 2-carbon donor and acceptor aldehyde apparently being met through the action of phosphoriboisomerase which brings about the interconversion of ribulose-5-phosphate and ribose-5-phosphate. This enzyme which has been highly purified from alfalfa by Axelrod & Jang (37) acted only on ribose-5-phosphate out of a number of compounds tested. The preparation, which is sensitive to sulfhydryl inhibitors and to phosphoribonic acid, had a turnover number of about 200,000 in the purest state attained. The equilibrium constant which is temperature dependent varies from 0.323 at 37° to 0.164 at 0°C. Its activity can be measured by following the rate of formation of ribulose-5-phosphate from ribose-5-phosphate by the Dische-Borenfreund carbazole test modified by the application of mild heating. The activity of this enzyme is very high in crude alfalfa and spinach juice; Horecker *et al.* (34) report the isomerase to be present even in highly purified spinach transketolase.

Transaldolase.—Transaldolase, discovered by Horecker & Smyrniotis (38) brings about the transfer of a 3-carbon piece from the heptulose-7-phosphate to the triosephosphate acceptor arising from carbons 3, 4, and 5 of the pentose phosphate. Fructose-6-phosphate is produced as a result (reaction 6 in Fig. 1). This reaction explains the observation that hexose was a product of pentose phosphate metabolism by cell free preparations from plants.

The fate of the 4-carbon residue from heptulose phosphate, which was

found to be a very reactive tetrose phosphate, was made clear upon the demonstration that the transketolase enzyme was capable of using this as an acceptor for the "active glycolaldehyde" from carbons 1 and 2 of the pentose phosphate. The product of this condensation (reaction 9 in Fig. 1) is again fructose-6-phosphate. It is thus clear that by the concerted action of these two enzymes all of the sedoheptulose carbon can be returned to hexose, and the sum of the reaction can be written:

Pentose phosphate + sedoheptulose phosphate → 2 hexose phosphate.

However, if the cycle is to be completed one must suppose that the triosephosphate residue resulting from the ribulose phosphate in the second transketolase reaction (reaction 9 in Fig. 1) is also capable of being converted, by means of triosephosphate isomerase and aldolase into fructose diphosphate. At least in those extracts which contain no other means for its removal this seems to be its fate.

The enzyme transaldolase has not been purified from plant materials, but its action and the associated transketolase action mentioned above can be logically inferred to have occurred from the results of some elegant experiments of Gibbs & Horecker (39). These workers isolated the glucose-6-phosphate produced from specifically labeled pentose phosphate by aqueous extracts of acetone powders prepared from pea tissues. They then degraded the glucose moiety of the glucose-6-phosphate to ascertain the distribution of the C^{14}. When ribose-1-C^{14} was the substrate for the pea root preparation the radioactivity was found almost exclusively in carbons 1 and 3 of the glucose, with 70 per cent in C-1 and 29 per cent in C-3. If the origin of the glucose had been solely by sedoheptulose formation and subsequent transaldolase reaction, equal labeling would have been expected in C-1 and C-3. However, if this reaction were occurring at the same time as the active glycolaldehyde (containing the radioactive C-1) from the pentose phosphate was being transferred by transketolase to the tetrose phosphate as shown in Figure 2, a second kind of glucose labeled only in C-1, would result. Since any hexose formed from carbons 3, 4, and 5 of the pentose phosphate would be unlabeled, the net expectation, if the reactions were exactly as suggested would be glucose with 67 per cent of its activity in C-1 and 33 per cent in C-3. The observed labeling is in good agreement with this expectation. Confirmatory evidence was provided by experiments with ribose-2-C^{14}, in which the labeling was again substantially as predicted by the above reactions. It was further found that when pea leaf preparations were used in similar experiments with ribose-1-C^{14} there was a small but significant amount of activity in C-4 and C-6. Figure 2 represents the passage of six glucose-6-phosphate molecules through the pentose phosphate cycle. The net result is that one molecule of glucose-6-phosphate is converted to six molecules of CO_2.

Evidence for the Pentose Phosphate Pathway *in Vivo*

The demonstration that enzymes can be extracted from plant tissues which catalyze the reactions shown in Figures 1 and 2 immediately raises the

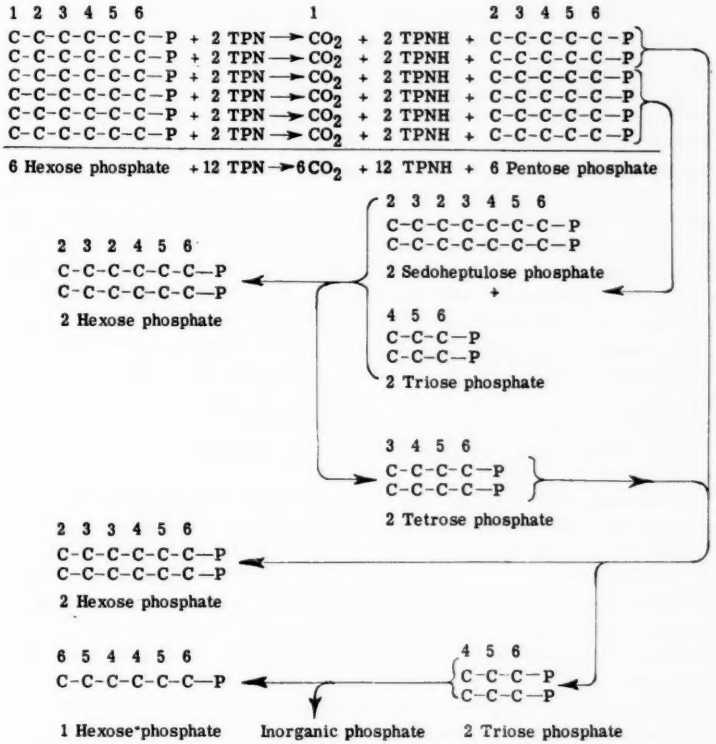

1 2 3 4 5 6 + 2 TPN → CO2 + 2 TPNH + 2 3 4 5 6
6 Hexose phosphate + 12 TPN → 6 CO2 + 12 TPNH + 6 Pentose phosphate
2 3 2 3 4 5 6
2 Sedoheptulose phosphate
2 3 2 4 5 6
2 Hexose phosphate
4 5 6
2 Triose phosphate
3 4 5 6
2 Tetrose phosphate
2 3 3 4 5 6
2 Hexose phosphate
6 5 4 4 5 6
1 Hexose phosphate
Inorganic phosphate
4 5 6
2 Triose phosphate

FIG. 2. Schematic representation of the passage of 6 molecules of hexose phosphate through the pentose phosphate cycle. The numbers refer to the positions of the carbons in the original hexose.

question of whether such a sequence operates in the living cells. In the previous sections on reactions *in vitro*, we have for convenience, and as a concession to custom, discussed the enzymes normally thought of as "belonging" to the EMP sequence as a separate group and also described the pentose phosphate pathway as an autonomous cycle. However, it is fully realized that this assignment of enzymes to individual reaction sequences and the choice of direction of progress of the reactions shown in Figure 1 are arbitrary. In considering what might be the position *in vivo* one has to take into account the fact that these two pathways may not be completely independent or mutually exclusive, since there are intermediates (fructose-6-phosphate, fructose-1, 6-diphosphate, triose phosphate) and enzymes (aldolase, triosephosphate isomerase, hexosephosphate isomerase) common to them

both. It is still conceivable, however, that, although the enzymes of the two pathways generally appear to be in the soluble condition, they may be spatially separated in the protoplasm so that they act on different carbohydrate pools. The EMP sequence has for a good many years been accepted as "the" pathway of glucose catabolism. Evidence of the participation of other sequences presumably can be gained from a variety of experiments. Not all of the possibilities have been exhausted in the results reported to date.

Evidence from experiments with inhibitors.—Unfortunately the properties of the enzymes of the pentose phosphate pathway which have been described do not include any suggestion that they are sensitive to inhibitors which would not at the same levels affect other enzymes concerned with glucose breakdown. Lundsgaard (40) used the so-called "differentiating effect" of iodoacetate on aerobic respiration and anaerobic respiration (fermentation) in yeast and muscle as evidence in favor of an alternative pathway of glucose breakdown in air. Turner (41) demonstrated a much less well-marked differentiating effect in carrots. It does not seem possible that an unequivocal conclusion can be drawn from the iodoacetate experiments. The concept of the alternate pathway has, however, received experimental support of other kinds of late.

Occurrence of the intermediates of the pentose phosphate pathway in vivo.—Analysis of respiring tissues, particularly by the improved techniques which have recently become available, may be expected to furnish at least supporting evidence for steps of pentose phosphate oxidation *in vivo*. It may be possible, for instance, to show that some of the intermediates are normally present in the tissues and that they fluctuate in amount in response to a change from air to nitrogen or on feeding likely precursors. The judicious use of labeled compounds is clearly of value here, and most of the evidence we have that compounds such as pentose phosphate, ribulose diphosphate, and sedoheptulose phosphate are in fact normal constituents of plant cells and that they are subject to metabolic transformations comes from work with $C^{14}O_2$ [Benson *et al.* (42)].

Intermediates of the pentose phosphate pathway as respiratory substrates.—Information of considerable value can be obtained from simple experiments in which it is ascertained whether compounds thought to be intermediates in the pentose phosphate cycle are in fact rapidly respired to CO_2 when supplied to tissues. The use of labeled intermediates allows a decision to be made as to whether the compound is respired even though no increase in the rate of respiration may be brought about.

Tolbert & Zill (43) using sedoheptulose-C^{14} which they had isolated from *Sedum* provided with $C^{14}O_2$ (44) were able to show that this compound is actively metabolized in sugar beet, tobacco, and barley leaves (see below). Corn coleoptiles can also utilize this compound as respiratory substrate both in air and nitrogen [Beevers (45)]. A variety of plant materials, including carrot root discs, pea and corn roots, pea internodes, and corn coleoptiles have now been shown [Beevers (45)] to be capable of using the pentoses D-

xylose-1-C^{14} and D-ribose-1-C^{14} (but not D-arabinose-1-C^{14}) as respiratory substrates. It will be noted here and in other experiments discussed below that D-xylose exhibits a parallelism in behavior to D-ribose which suggests that it is metabolized by a very similar mechanism.

Ribose and xylose were also utilized in respiration under nitrogen. The interesting fact emerges that C-1 of each of the pentoses appears both in the alcohol and in the CO_2 produced under these conditions. Degradation of the alcohol showed that the radioactivity was very largely in the methyl group, in which the activity was roughly twice that in the CO_2. Such a distribution of activity would have resulted if derivatives from the supplied pentoses had been converted by the transketolase and transaldolase sequence of reactions (Figs. 1 and 2) into hexose or triose which in turn would have given rise to pyruvate by the EMP reactions. The labeling in pyruvate so derived from C-1 labeled pentoses would be expected to be 66 per cent in CH_3 and 33 per cent in the COOH carbon, and its conversion to alcohol and CO_2 would yield products labeled in the manner actually observed. The distribution of label in the hexose produced from pentose phosphate-1-C^{14} in cell free extracts was found by Gibbs & Horecker (39), as noted above, to be consistent with the operation of this same sequence.

On the basis of what is known about the general nonreactivity of the free sugars in the enzyme sequences which have been studied, one may surmise that the ready utilization of compounds such as sedoheptulose, D-ribose, and D-xylose (and other compounds mentioned in the next section) is preceded by phosphorylation in the tissues. There is no lag period comparable to that found in the utilization of the pentoses in some bacteria, which must first be adapted to the substrate, and it may therefore be presumed that, in the materials studied, the requisite kinases are already present.

Conversion of intermediates of the pentose phosphate cycle to other compounds in living tissues.—In some experiments, C^{14}-labeled compounds which are closely related to members of the cycle have been introduced into plant tissues, usually leaves, by infiltration or by allowing them to be taken up in the transpiration stream. Analysis for products isolated after varying periods of time have shown that such compounds are actively metabolized by the tissues. Some of the results point clearly to the operation of a sequence of reactions similar to that shown in Figures 1 and 2. For instance, Tolbert & Zill (43) made chromatographic separations of compounds produced after supplying sedoheptulose uniformly labeled with C^{14} to barley, sugar beet, and tobacco leaves. In the light, in the presence of air, sedoheptulose carbon was rapidly incorporated into sucrose, and only small amounts of other compounds appeared. Chromatographic analysis of the phosphate esters which were formed indicated the presence of radioactive erythrose and erythulose. Under nitrogen in the light the conversion to sucrose was drastically reduced, but other compounds particularly alanine, were found in considerably larger amounts than those observed in the air experiments. Radioactive ribulose, ribose and fructose, and two unknown "sugar acids"

were also detected in the hydrolysis products of the phosphate esters. These authors point out that such products are characteristic of the pentose phosphate cycle and of the photosynthetic carbon cycle.

It is also important, in view of the possibly complicating effect of light, that sedoheptulose was also shown to be utilized, to some extent at least, in the dark and converted to similar products, although the phosphate esters were not separately analyzed in this case. From anaerobic experiments in the dark in which no radioactive sucrose or other compounds except alanine were detectable the authors conclude that "the alternate sugar metabolism cycle is aerobic" (43). It should be pointed out, however, that as shown in Figure 1 the pentose phosphate oxidation cycle is aerobic only insofar as re-oxidation of the nucleotides reduced in the glucose-6-phosphate and 6-phosphogluconate dehydrogenations are normally thought of as involving electron transfer to O_2, and that no oxidative steps occur between sedoheptulose phosphate and sucrose.

As these authors point out, no attempt was made to measure the volatile and gaseous products of sedoheptulose utilization, and it is possible that in the N_2 experiments at least some of the substrate was converted to triose and pyruvate and thence to alcohol and CO_2 which would be undetected. The fact that alanine was found as the only radioactive product when sugar beet leaves were provided with sedoheptulose-C^{14} in the dark in N_2 might be in accord with this suggestion. It has now been shown that significant amounts of labeled alcohol and CO_2 are produced from sedoheptulose-C^{14} when it is supplied to corn coleoptiles under strictly anaerobic conditions in the dark [Beevers (45)]. The ready utilization of pentoses in similar experiments also indicate that an aerobic condition is not a requirement for the operation of at least some of the steps of the pentose phosphate cycle. If there were some internal electron transfer which could be linked through TPNH to the oxidation of glucose-6-phosphate and 6-phosphogluconate there seems no *a priori* reason why the complete cycle might not operate under N_2.

In some interesting experiments with wheat Brown & Neish (46) and Neish (47) fed D-ribose-1-C^{14}, D-xylose-1-C^{14} and sedoheptulose-2-C^{14} and after 5 to 48 hr. isolated cellulose and xylan from the plants. Both the pentoses and the sedoheptulose served as precursors for the glucose units of the cellulose and the pentose units in the xylan. (See also "Xylan formation" below). Degradation of the glucose (obtained by hydrolysis of the cellulose) revealed that a considerable spreading of the original C-1 of the pentose had occurred. Sedoheptulose-2-C^{14} gave rise to glucose units in which C-2 and C-5 were more or less equally labeled and together comprised 72 per cent of the activity. Xylan in which C-2 and C-5 of the xylose each contained 43 per cent of the activity was also formed. This distribution suggests that carbons 1, 2, 3 of the sedoheptulose give rise to a triose unit which becomes carbons 1, 2, and 3 of fructose-6-phosphate in the transaldolase reaction. The fructose-6-phosphate would then be phosphorylated to fructose-1,6-diphosphate. This compound then would be subject to aldolase and triosephosphate iso-

merase action with the consequent equalization of C-2 and C-5. The glucose, or more likely a derivative thereof, would lose its C-6 to produce the 5-carbon sugar. The labeling found in the glucose produced from the pentoses is roughly that expected if these compounds had first been converted by transketolase into sedoheptulose as an intermediate. It may be noted that these experiments were carried out in the light for rather long periods and that a certain amount of spreading of label into other positions may have been expected on this account.

Krotkov *et al.* (48) in recent experiments, also carried out on wheat but with uniformly labeled sugars, have demonstrated ready interconvertibility of some pentoses and hexoses. Ginsburg & Hassid (49) observed that in wheat seedlings D-xylose-1-C^{14} was converted into sucrose, of which the glucose moiety had the following distribution of radioactivity: C-1, 50 per cent; C-2, 2 per cent; C-3, 20 per cent; C-4, 8 per cent; C-5, 1 per cent; and C-6, 19 per cent. They point out that this type of labeling would have been expected if the xylose had been metabolized by the transketolase and transaldolase sequence of reactions with some randomization between C-1 and C-6 and between C-3 and C-4. The conclusions about the pathway of pentose utilization are thus similar to those reached from other recent experiments with D-ribose and D-xylose, and the authors point out the close parallel with the results in cell-free extracts observed by Gibbs & Horecker (39). It will be noted that in contrast to the results of Neish there was no approach to complete equalization of the two halves of the hexose molecule.

Evidence based on the demonstration that essential enzymes are present.—It should be stressed that to demonstrate that a given enzyme can be extracted from a tissue is not to provide evidence that it is active *in vivo*, and the successful extraction of an intact enzyme sequence does not in itself make the case for participation any stronger. Nevertheless, there have been several reports recently, based on such evidence, that the pentose phosphate pathway operates in such organisms as flies (50), worms (51), and bacteria (52), and indeed some workers have felt justified in making estimates of the participation of this pathway which were based solely on the amounts of two enzymes that they were able to extract (53).

Clearly the most that can be deduced from the fact that an enzyme is present is that it is potentially of significance *in vivo*. Inferences about the quantitative capabilities of an enzyme *in vivo* based on the amounts extractable can not be extended to an enzyme sequence of which this one is a part; such inferences are in addition subject to the reservation that the conditions for its action *in vivo* must not be less suitable than those under which its activity was measured *in vitro*.

Earl & Gibbs (54) have shown that, in the case of *Avena* coleoptiles, activities of the first enzymes of the pentose phosphate pathway, the glucose-6-phosphate and 6-phosphogluconate dehydrogenases, were higher than those of the EMP enzymes which were measured, although there is clear evidence that the respiration of glucose in this tissue is by way of the EMP pathway.

It should be mentioned also that the coleoptile tissue was among the most active of those tried, in utilizing pentoses [Beevers (45)]. The manner of utilization indicated that the enzymes of the pentose phosphate cycle subsequent to pentose formation were also present and potentially active. Clearly, attempts to assess these contributions must be directed to the living cells or tissues themselves. Furthermore, it must be recognized that the contributions of the different pathways depend on the substrate employed.

Techniques using specifically labeled glucose as respiratory substrate.— Bloom & Stetten (55) introduced a valuable method for demonstrating the participation of the pentose phosphate pathway in glucose catabolism. This technique takes advantage of the fact that glucose molecules which are subject to oxidation and decarboxylation to pentose phosphate give rise to CO_2 whose origin is the C-1 of the glucose, whereas, from molecules metabolized by way of the EMP sequence (which includes equilibration at the triose level and hence makes equivalent carbons 1 and 6, 2 and 5, and 3 and 4, respectively) the CO_2 would be initially richest in carbons 3 and 4, and carbons 1 and 6 would appear later and at equal rates. Thus, when equal samples of tissue are provided respectively with equal amounts of glucose-1-C^{14} and glucose-6-C^{14} the CO_2 outputs in the two cases are the same, but that from the glucose-1-C^{14} would be expected to be more radioactive than that from glucose-6-C^{14} if the supplied glucose was metabolized by way of the pentose phosphate pathway. They suggested that the ratio

$$\frac{\text{per cent radiochemical yield from glucose-6-C}^{14}}{\text{per cent radiochemical yield from glucose-1-C}^{14}}$$

the so-called C-6/C-1 ratio might be used to give directly the maximal fraction of the glucose which was respired by the EMP sequence. It should be emphasized that if the glucose were completely respired the final value of this ratio would be 1 regardless of the pathway of breakdown, and it is only during the initial stages of glucose utilization that the contribution of C-1 might be expected to exceed that of C-6. The C-6/C-1 ratio can not be regarded as an unchanging value typical of a particular tissue, and furthermore, as pointed out by Beevers & Gibbs (56), it cannot be assumed that all tissues will have the same relative abilities in carrying out the reactions subsequent to and prior to C-1 release, so that, even if all of the respiration were occurring by the pentose phosphate pathway, the ratios obtained from different tissues might not be the same. An additional possibility is that in some tissues the reactions subsequent to 6-phosphogluconate decarboxylation which result in the appearance of C-6 in the CO_2 may be so rapid relative to those bringing about the release of C-1 that a value close to 1 for the C-6/C-1 ratio might be obtained even though the contribution of the EMP pathway may be small. However, in some cases where ratios close to 1 have been observed there is other evidence to support the idea that the "maximum contribution of EMP" represented by the ratio near unity, is in fact the actual one.

Horecker & Mehler (21) emphasize the need for great caution in interpreting the results from labeled precursors "in view of the complicated pattern of carbohydrate interconversions which are possible" under the influence of enzymes now known to be present. Certainly it now appears an oversimplification to regard the two well-established alternative pathways as mutually independent, and it may be more appropriate to regard the pentose phosphate pathway as an alternative route to the triose phosphate (and thence to pyruvate) with only some of the pentose carbon being converted back to glucose and recycled. However this may be, the key reaction which can be used to distinguish between the two pathways of glucose-6-phosphate breakdown is the early release of C-1 of the glucose into the respired CO_2 in the pentose phosphate pathway. The predominance of this carbon over others and particularly over that of C-6 observed in experiments with specifically labeled glucose may be accepted as evidence of the participation of at least the initial reactions of the pentose phosphate pathway, if not of the complete cycle itself, in the respiration of the tissue concerned.

The implications of some recent work of Edelman, Ginsburg & Hassid (57) should also be mentioned here. These workers have shown that in wheat tissues 15 per cent of the added glucose-1-C^{14} may be converted into glucose-6-C^{14} in 30 min. Since a corresponding conversion of C-6 labeled glucose into C-1 labeled glucose occurs, the authors suggest that the randomization is attributable to a combination of aldolase and triosephosphate isomerase activity on the fructose diphosphate in equilibrium with the glucose. If this reaction occurs to a significant extent in short-term experiments with other tissues, it is obviously important to consider what influence it might have on the evaluation of C-6/C-1 ratios. Firstly, it should be clear that whatever the extent of this reciprocal transformation, the ratio expected from the operation of the EMP sequence would still be unity. A simple calculation will show that the influence of such a reaction in a tissue in which a portion of the respiration was by way of the pentose phosphate pathway is to lead to C-6/C-1 values somewhat higher than those obtained if the randomization was not occurring. Thus the result of this complicating reaction would be an underestimation of the part played by the pentose phosphate pathway.

When resting yeast suspensions were examined by the Bloom & Stetten method, Beevers & Gibbs (58) showed that there was a clear initial predominance of C-1 in the respired CO_2 indicative of the participation of the pentose phosphate pathway. Measurements of the relative contributions of C-1 and C-6 were continued until all of the supplied glucose had been utilized (by respiration or assimilation) and it was found that in the later stages, the CO_2 was richer in C-6 than C-1, so that eventually the total yield from C-1 was the same as that from C-6. This demonstrated that no preferential assimilation of a 5-carbon unit derived from glucose by loss of C-1 had occurred to influence the conclusions in the direction of an overestimation of the participation of the pentose phosphate pathway in respiration.

Beevers & Gibbs (56) also applied the Bloom & Stetten procedure to

higher plant tissues. Out of a variety of materials tested, and in contrast to the results by Bloom & Stetten (55) from animal tissues, the majority yielded ratios considerably less than unity, and only in one case, that of the corn root tip, was a value close to 1 observed. This was a tissue from which Beevers & Gibbs (59) obtained evidence by an independent method that the EMP pathway is by far the most important both in nitrogen and in air. In this method the tissue was fed various specifically-labeled glucoses in the presence of arsenite. The arsenite retarded the oxidative consumption of pyruvate and led to an accumulation of ethanol. The distribution of the radioactivity in the ethanol was found to be as expected if the pyruvate had been produced solely by the EMP sequence. In later experiments Gibbs & Beevers (60) showed that other meristematic and undifferentiated tissues such as root tips of other species and *Avena* and corn coleoptiles gave C-6/C-1 ratios close to unity, whereas older, and particularly aerial tissues again provided ratios which indicated a very significant contribution of the pentose phosphate pathway. In every case in which "old" and "young" tissues of the same type were compared, such differences as were observed indicated a greater participation of the pentose phosphate pathway in the older material. In none of these experiments was there any direct demonstration that assimilation of a 5-carbon fragment was not occurring and leading to an apparently low value for the C-6/C-1 ratio. However, to the extent that those tissues (i.e., meristems) in which such a reaction might have been expected to be occurring to the greatest extent were the very ones which gave values close to unity (indicating the minimum operation of the pentose phosphate pathway) the ratios observed with the various tissues may be assumed to be little influenced on this account. Cohen (60a) has presented a critical and valuable discussion of the problems in evaluating carbohydrate pathways.

Finally, indications that a pathway other than the EMP pathway is operating may come from experiments in which a labeled intermediate of this sequence, on being introduced into the tissue, is not respired and does not suffer dilution. Such an alternative pathway may also be suspected if it is found that a known intermediate of the EMP sequence, though present in the tissue, does not respond in the expected way when large changes in the respiratory rate are induced. A case in point is the "pyruvate anomaly" described by Barker & Mapson (61). In potatoes transferred from 10° to 1°C. the pyruvate level in the tissues remained unchanged, while there were some striking changes in CO_2 output and evidence of starch or sugar utilization. It is, of course, possible that the pool of available pyruvate through which turnover was occurring represented a small fraction of that actually shown to be present, but the authors are inclined in this case to regard the results as at least suggesting the operation of an alternate sequence of respiratory carbohydrate breakdown which does not include pyruvate as an intermediate.

The situation when light is applied to a green tissue previously respiring

in the dark is also an interesting one in this regard. Calvin & Massini (62) and Gibbs (63) have presented evidence that the operation of the Krebs cycle is suspended or at least very much curtailed when the light is supplied to green tissues, although, as Brown (64) has shown unequivocally in beautiful experiments with oxygen isotopes, the rate of O_2 uptake is generally unaffected by the transition from dark to light conditions. It seems plausible that the photolysis of water in the light reaction furnishes the alternate source of electrons when flow from the Krebs cycle is restricted. A further suggestion which might be considered is that when the Krebs cycle is slowed down more glucose is diverted to the pentose phosphate pathway [Stiller (65)]. Then the CO_2 output and glucose consumption would also continue at undiminished rates in the light.

Mechanism of the Aerobic Consumption of Pyruvate in Plants

It is not intended here to discuss in detail the progress in this area of endeavor, particularly since the cell-free aspects of this problem have been covered in a recent survey by Hackett (66). A brief summary of the present position is given. Whatever the pathway of its formation, pyruvate is presumably subject to oxidative attack by the same enzyme system.

For those who, on the basis of comparative biochemistry and some indirect evidence, were inclined five years ago to implicate the Krebs cycle as the mode of pyruvate oxidation in plants, the intervening period has provided very encouraging, if not overwhelming, confirmatory evidence, Since the reviews of Millerd & Bonner (67), Burris (68), and Goddard & Stafford (69) which summarized the early progress in this direction, several other reports have appeared of the isolation from a variety of tissues of active particulate preparations capable of inducing oxidation of Krebs cycle acids [see Hackett (66, 69c), Tager (69a), Abramsky & Biale (69b), Switzer, Smith & Loomis (70), Kmetec & Newcomb (71), Beevers & Walker (72, 73)]. However, the situation in some green leaves requires further study. In lupine, several of the enzymes which are normally regarded as mitochondrial were found only in the soluble phase of the leaf homogenate by Brummond & Burris (74) and in mature *Bryophyllum* leaves Stutz & Burris (75) showed that there is evidence only of a sluggish pyruvate oxidation (Krebs) cycle.

Beevers & Walker (72) have shown that high and sustained rates of oxidation of every member of the cycle are brought about when an appropriate supply of co-factors, including coenzyme A and cocarboxylase, is provided to preparations from castor bean endosperm. The requirements for pyruvate oxidation have been more closely defined in mitochondria from this tissue [Walker & Beevers (73)] and from avocado [Abramsky & Biale (69b)]. It is also noteworthy that Funahashi *et al.* (76) and Seifter (77) have provided further evidence on the occurrence of coenzyme A in a variety of tissues. Several recent reports describe phosphorylations associated with the oxidation of individual acids [Lieberman & Biale (78), Kmetec & Newcomb (71), Switzer *et al.* (70)] and of intermediates in electron transfer [Fritz & Naylor

(79)]. While the P/O values obtained are frequently considerably less than theoretical, lupine [Conn & Young (80)] and castor bean [Akazawa & Beevers (81)] have yielded mitochondria giving P/O ratios for α-ketoglutarate oxidation of greater than 3.0. There is growing reason to believe that this process is not necessarily less efficient in mitochondria from plants than from other sources.

The net effect of the recent activity in this field has thus been to emphasize that plant mitochondria, properly treated, conform in general to the pattern established for pyruvate oxidation by animal mitochondria. However, a divergence from the animal system has been noted by Kaufman & Alivisatos (82) and Kaufman (83) who found that the reaction in which the substrate level phosphorylation accompanying α-ketoglutarate oxidation occurs as follows in spinach:

$$\alpha\text{-ketoglutarate} \xrightarrow[\text{CoA}]{\text{DPN}} \text{succinyl-CoA}$$

$$\text{succinyl-CoA} + \text{inorganic phosphate} + \text{ADP} \rightarrow \text{succinate} + \text{CoA} + \text{ATP}$$

This is in contrast to the pig heart system which Sanadi *et al.* (84) showed used inosine diphosphate or guanine diphosphate, but not ADP, as the primary acceptor.

Lehninger (85) has shown that external pyridine nucleotides can be oxidized by mitochondria, with concurrent phosphorylation. Thus, the oxidative machinery of the insoluble mitochondria can perform the energetically useful oxidation of the reduced nucleotides which are formed by the soluble dehydrogenases of the carbohydrate sequences.

Other Transformations of Carbohydrates

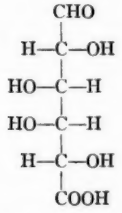
Although it is beyond the scope of this review to delve into the problem of polysaccharide formation *per se* in higher plants, there are certain recent findings which do not prove, but do imply the existence of carbohydrate intermediates and carbohydrate transformations which may not be unimportant in considering solutions to the general problems of carbohydrate breakdown. Certain miscellaneous reactions discussed below are of interest for the same reason.

Pectin formation.—A preliminary examination of the formation of pectin *in vivo* from specifically labeled glucose [Axelrod, Seegmiller & McCready (86)] strongly suggests that the galacturonic acid and D-arabinose associated with pectin arise directly from the glucose skeleton. In experiments performed with boysenberry fruit it appeared that galacturonic acid is derived directly from glucose, since C-1 labeled glucose led to predominately C-1 labeled galacturonic acid. A secondary finding which may be important in the future consideration of glucose catabolism is that carbons 2 and 5 turned out to be somewhat highly and equally labeled, whereas 3, 4, and 6 were only poorly labeled. The breakdown and recombination (presumably superimposed on the main hexose to galacturonic acid conversion) is of a type not predictable by the conventional EMP or pentose phosphate transformations.

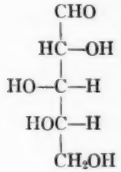
XUM

Another conclusion was that the number 6 carbon undergoes conversion to carboxylic acid.

The L-arabinose (derived from the araban associated with the pectin) was predominantly labeled in the number 1 position implying the loss of carbon 6 from the original hexose chain. For once, at least, one observes a triumph of paper and pencil chemistry; what a carbohydrate chemist would have been inclined to predict, holds true. An examination of the structural formula of D-galacturonic acid and L-arabinose indicates their close relationship.



D—Galacturonic Acid



L—Arabinose

This mode of pentose formation must involve reactions distinct from those of the pentose phosphate pathway. These reactions remain to be elucidated. In this connection the recent finding by Rabinowitz (87) that rat liver and kidney preparations decarboxylate glucuronic acid and its lactone is most interesting. The stimulatory effect of UTP, among other co-factors, may suggest the involvement of a uridine diphosphoglucuronate intermediate.

Xylan formation.—Closely related to the above observations is the formation of xylan in wheat. Neish (47) found that the D-xylose obtained from the xylan of the cell wall of wheat which had been presented with specifically-labeled glucose, was labeled in accord with the supposition that it was derived from hexose by loss of the number 6 carbon. On the other hand, as noted above, isotopic experiments with ribose and xylose indicated that they did not give rise to xylans without first being transformed to hexose. Ginsburg & Hassid (49), also studying the behavior of wheat seedlings, presented evidence that the pentose components of the hemicellulose arose by loss of the number 6 carbon of the hexose.

Cellulose synthesis.—That under some conditions there can be a very efficient transformation of glucose into a polysaccharide, *in vivo*, was demonstrated by Greathouse (88) who introduced C-1 labeled glucose via the stem to an isolated cotton boll 21 days after pollination and obtained almost 50 per cent conversion of glucose into cellulose. It is of special interest that the glucose derived from the cellulose was labeled exclusively in carbon 1. Thus under these conditions degradation and recombination of glucose did not occur.

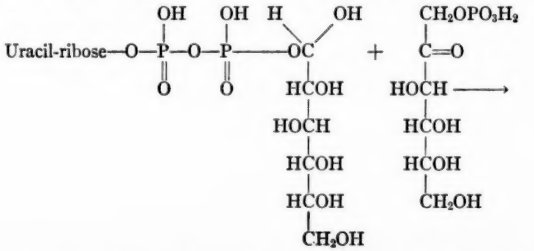
Sucrose synthesis.—Although the synthesis of sucrose by plants has long occupied the attention of plant scientists, it is only in the past several years

that any success has been attained with cell-free preparations. The earlier studies of Nelson & Auchincloss (89) with potato slices established that either glucose or fructose could give rise to sucrose provided air was present and showed further that photosynthesis was not essential to the process. These observations were extended to other plants and organs by Virtanen & Nordlund (90), McCready & Hassid (91), and Hartt (92). With the recognition by biochemists that energy was required for the formation of glycosidic bonds, and with the knowledge that glucose and fructose were interchangeable as precursors, it became natural to suspect that hexose phosphates were intermediates, with respiration required to generate the phosphate bonds. Indeed, the discovery by Doudoroff, Kaplan & Hassid (93) of sucrose phosphorylase might have seemed a solution to this problem. The reaction catalyzed by this enzyme is represented as follows:

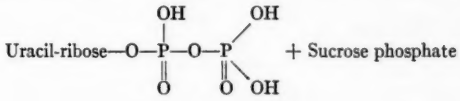
$$\text{glucose-1-phosphate} + \text{fructose} \rightleftharpoons \text{sucrose} + \text{phosphate}.$$

In this instance the principle of economy of comparative biochemistry was badly violated for the enzyme was found to be confined to a small group of microorganisms. After an exhaustive search, Hassid (94) concluded that this enzyme was not responsible for sucrose synthesis in plants.

The elucidation of sucrose synthesis in plants resulted from an interesting sequence of events, many of which were not directed to a solution of this problem. Calvin & Benson (95), in studying the path of carbon in photosynthesis, noted that the first free sugar after presenting $C^{14}O_2$ was sucrose and that glucose and fructose appeared later. From this they concluded that monoses were first present as phosphorylated sugars and accordingly suggested that sucrose might first appear as a phosphorylated compound. Ultimately Calvin's group (96), and particularly Buchanan (97), isolated a substance from a short-time photosynthesis experiment with sugar beet which had the properties of sucrose phosphate and yielded a fructose phosphate on hydrolysis. Unknown nucleotides containing glucose were also among the products. Thanks to the previous discovery and description by Caputto *et al.* (98) of UDPG, the coenzyme for phosphogalactoisomerase, the Calvin group was readily able to demonstrate this compound among their unknown nucleotides. Then with a remarkable display of insight they suggested that the reaction by which sucrose was formed might occur as follows:



Uridine diphosphoglucose Fructose-1-phosphate



Uridine diphosphate

Kinetic experiments by Benson *et al.* (99) showed that the rate of $C^{14}O_2$ fixation into uridine diphosphoglucose was more rapid than into the sucrose, which was in harmony with the proposed route. Following this suggestion and forearmed with the successful synthesis of trehalose phosphate [Leloir & Cabib (100)] (1, 1-α-glucose-α-glucoside) from uridine diphosphoglucose +glucose-6-phosphate using a yeast enzyme, Leloir and his Buenos Aires group (101, 102) succeeded in demonstrating the synthesis of sucrose in a plant extract from uridine diphosphoglucose plus fructose. It is noteworthy that this system did not utilize fructose phosphate. Two alternate names, "UDPG-fructose transglycosylase" and "saccharese" have been proposed for this enzyme by the discoverers. The "ese" suffix was chosen in accord with the old suggestion of von Euler (103) that it be employed to indicate synthesizing enzymes in contrast to hydrolytic enzymes. Without questioning the prerogative of the discoverers to name their discovery, the reviewers must express their strong preference for the first name. Most of the synthesizing enzymes catalyze reversible reactions; the nomenclature "ese" thus freezes a point of view into a name. Further examination of plant extracts by Leloir & Cardini (104) did indeed reveal the existence of an enzyme which utilized fructose-6-phosphate in preference to fructose and formed sucrose monophosphate, in which the phosphate was apparently attached to the 6 position of the fructose moiety. It will be noted that this was at variance with Buchanan's postulation of a fructose-1 linkage. In accord with the above nomenclature the second enzyme might be called "UDPG-fructose-6-phosphate transglycosylase." The first enzyme was found in wheat, corn, and bean germ, in potato sprouts, in germinated beet, sweet sorghum, and pea, in sugar cane and barley shoots and sugar cane roots, and in pea, pine, and fenugreek seeds. The second enzyme was present in wheat germ.

At first glance it might seem that the relative importance of these two modes of synthesis could be assessed *in vivo* by introducing one of the labeled monoses and then noting the distribution of label between the glucose and fructose moieties. Thus, if labeled glucose were introduced and the UDPG-fructose transglycosylase enzyme were operative it might be expected that some of the endogenous fructose would appear in the fructose moiety of the sucrose. On the other hand, if the fructose-phosphate transglycosidase were involved, the fructose and glucose in the sucrose would be equally labeled because, first of all, there is a very limited amount of endogeneous fructose-6-phosphate present and, secondly, the ubiquitous hexose isomerase should make fructose-6-phosphate readily interchangeable with glucose-6-phosphate. The glucose would necessarily have to be phosphorylated before it

became incorporated in UDPG if the results of Munch-Petersen *et al.* (105) with yeast are applicable here.

The evaluation of such experiments is complicated by the probable existence of the endogenous monoses in metabolically sluggish pools and by the possibility of transglycosidation by invertase. Perhaps all that can be said at present is that equality of labeling between the two halves of sucrose would not rule out the possibility of the UDPG-fructose-6-phosphate transglycosylase. Although equality of labeling in the sucrose formed when the plant tissue received either glucose or fructose has been shown in some cases [Hassid & Putman (106)], there have been a number of examples in which the contrary has been observed. For example, it was found by Axelrod & Seegmiller (107) that in experiments with apple tissues five times as much activity appeared in the glucose as in the fructose moiety, when labeled glucose was available under anaerobic conditions; under aerobic conditions the ratio was 2.5. Putman & Hassid (108) found a small but nonetheless significant disparity in distribution in canna leaf discs, with labeled glucose or labeled fructose. Vittorio *et al.* (109) presented data which indicated nonuniform distribution in anaerobic experiments with tobacco leaves, although no difference was observed in aerobic experiments using labeled glucose.

The results of some most interesting experiments of Porter & May (110) in which radioactive glucose, fructose, and sucrose were supplied to leaf discs support the idea of the ready interconvertibility of glucose or fructose, once they are absorbed, although the monoses are not taken up at equal rates. They also find support for the concept of metabolically distinct pools of the sugars within the tissue. An unexplained result of considerable interest to the problem of carbohydrate transformation is that the presence of CO_2 promotes the accumulation of sucrose, while it depresses that of starch, but has no influence on the amount of carbohydrate uptake.

Erythulose phosphate.—Mueller *et al.* (111) have effected the condensation of triosephosphate with formaldehyde by homogenates of Swiss chard leaves. The product, 1-phosphoerythulose was labeled in the 4 position when radioactive formaldehyde was used. Although this reaction appears to be a consequence of the nonspecific action of aldolase, it should be noted that a similar reaction detected by Charalampous & Mueller (112) in liver extract has been attributed to an enzyme different from aldolase by Charalampous (113). The significance of this reaction *in vivo* remains to be demonstrated. Although triosephosphate is a normal metabolite, free formaldehyde is not ordinarily encountered.

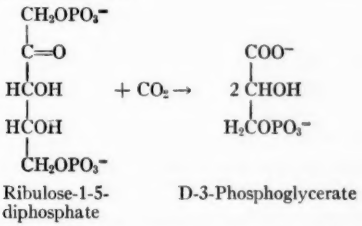
Mannoheptulose.—The 7-carbon ketose formed by the action of transketolase on ribulose phosphate and ribose phosphate was unequivocally identified by Horecker *et al.* (34) as sedoheptulose-7-phosphate. It is therefore of interest to inquire how mannoheptulose, the predominant heptulose of some avocado varieties, is formed, and to see if perhaps it is an alternate heptulose in the pentose phosphate pathway. While these answers are not yet on hand the experiments of Nordal & Benson (114) in short term photosynthesis

experiments with radioactive carbon dioxide have revealed that mannoheptulose monophosphate as well as sedoheptulose monophosphate are among the early products in the leaf. The rates of incorporation of radioactivity indicate the mannoheptulose phosphate to be the precursor of the mannoheptulose. That heptulokinase activity is present in plants can be inferred from the fact that sedoheptulose can be metabolized by them (see above).

Shikimic acid formation.—It is most interesting that two products of carbohydrate breakdown, D-erythrose phosphate and phosphoenolpyruvate can be condensed by the action of a cell-free extract of *E. coli* to form dihydroshikimic acid [Srinivasan *et al.* (115)]. Since dihydroshikimic acid is in the sequence leading to phenylpyruvic acid [Davis (116)], this reaction is of importance in aromatization. The reaction has not yet been observed in higher plants, but it will be recalled that shikimic acid occurs widely in the plant kingdom and was first discovered in *Illicium religiosium.* Brown & Neish (117) have recently shown that shikimic acid and phenylpyruvic acid can be incorporated into lignin in wheat and maple plants.

The *E. coli* extract mentioned above can also convert sedoheptulose-1, 7-diphosphate to dihydroshikimic acid. A dependence on DPN has been shown for this reaction. Srinivasan and his co-workers propose that the apparent mechanism of this reaction is first an aldolase cleavage of the sedoheptulose diphosphate into D-erythrose phosphate and dihyroxyacetone phosphate. The triose phosphate is then transformed to phosphoenolpyruvate by the conventional EMP pathway, the DPN being required for the oxidative step. The reversible condensation of erythrose-4-phosphate and dihydroxyacetone phosphate has been shown by Horecker *et al.* (118) to be catalyzed by aldolase.

Ribulosediphosphate carboxylase.—One of the most notable achievements in plant biochemistry in recent years has been the discovery of the ribulosediphosphate carboxylase reaction



Ribulose-1-5-diphosphate — D-3-Phosphoglycerate

The importance of this reaction lies in the fact that it provides the mechanism whereby CO_2 is fixed in photosynthesis. The carbon pathway in photosynthesis is thenceforth needed to convert the resulting phosphoglyceric acid to hexose, part of which regenerates pentose diphosphate. Virtually all of the enzymes needed for the operation of the pathway are also the enzymes of the so-called EMP pathway and the pentose phosphate pathway. The treatment of these nexi of enzymes from the view point of photosynthesis will not

be presented here. The reader is referred to the recent summary of Calvin (119) for a comprehensive picture, as well as to the articles by Vishniac (120) and Arnon (121) in the *Annual Review of Plant Physiology.*

The discovery of ribulosediphosphate carboxylase represents the triumphant culmination of one phase of the systematic attack on the problem of photosynthetic carbon dioxide fixation launched by Calvin, Benson, and others in the Radiation Laboratory at the University of California. Through the skillful use of tracers, ribulose diphosphate was discovered in nature for the first time. Then, by the application of kinetic studies to the steady-state photosynthetic system, the California group was actually able to predict the existence of the carboxylase enzyme [Bassham *et al.* (122)]. The realization of this prediction was made by Quayle *et al.* (123) in the same laboratory, using cell-free extracts of *Chlorella* and naturally-produced ribulose diphosphate. Simultaneously Weisbach, Smyrniotis & Horecker (124) demonstrated CO_2-fixation in a cell-free extract of spinach containing ATP and ribulose-5-phosphate. It was subsequently established that this preparation contained a pentosephosphate kinase (see below). A moderate purification of the enzyme has been reported by Racker (125). He employed an indirect spectrophotometric assay for the enzyme based on the oxidation of DPNH by the 1,3-diphosphoglycerate derived from the 3-phosphoglycerate formed in the carboxylation reaction. The reaction mixture contained ribose-5-phosphate, phosphopentoisomerase, ATP, Mg^{++}, phosphoglyceric kinase, glyceraldehyde-3-phosphate dehydrogenase, and DPNH. These enzymes were present in excess.

Phosphopentokinase.—The requirement for ATP in the fixation of CO_2 by ribose-5-phosphate in spinach extract, as noted by Weisbach *et al.* above, taken together with the lack of such a requirement when ribulose diphosphate was the substrate, as demonstrated by Quayle *et al.* (123), pointed to the presence of a phosphorylating enzyme. Weisbach *et al.* (126) speedily proved that a phosphopentokinase was indeed present in the spinach extracts. When ATP, ribose-5-phosphate, and the moderately purified enzyme were incubated, a product was obtained, after ion-exchange chromatography, which contained two phosphate groups for each pentulose molecule. The dephosphorylated product appeared to be identical with ribulose when chromatographed on paper. The presence of phosphoriboisomerase made it impossible to decide whether ribose-5-phosphate or ribulose-5-phosphate was the phosphate acceptor. Racker has recently reported a somewhat improved purification of this enzyme (125). He has also introduced an assay method which is based on coupling with the acetokinase reaction:

$$\text{Pentose phosphate} + \text{ATP} \rightleftharpoons \text{ribulose-1,5-diphosphate} + \text{ADP}$$

$$\text{ADP} + \text{acetyl phosphate} \rightleftharpoons \text{ATP} + \text{acetate.}$$

The acetyl phosphate is present in excess in the reaction mixture together with an excess of the acetokinase enzyme. Under these conditions the rate of disappearance of the acetyl phosphate, which is readily measured by Lip-

mann & Tuttle's hydroxamic acid method (127), is a function of the concentration of the phosphopentokinase.

Xylulose phosphate formation.—Forrest *et al.* (128) demonstrated the formation of what was quite likely xylulose phosphate, from glycolaldehyde and triosephosphate under the influence of a pea preparation presumably as a result of its aldolase activity. McGeown & Malpress (129) since showed a similar reaction to be catalyzed with a liver extract. In both, ribulose was also detected, indicating an isomerase. However, the occurrence of glycolaldehyde in these tissues as a normal free constituent remains to be established. It is therefore most pertinent that Ashwell & Hickman (130) obtain D-xylulose phosphate upon treating ribose-5-phosphate, a naturally occurring metabolite, with mouse spleen extract. Hochster (131) described a similar enzyme in *Pseudomonas hydrophila*, and Dickens & Williamson (132) have shown this transformation to occur with rabbit muscle and horse erythrocyte preparations. These latter workers noted that in rabbit muscle extract, ribulose-5-phosphate-ribose—5-phosphate isomerase was also present, so that the final reaction mixture contained ribose, ribulose, and xylulose phosphates. With the horse erythrocytes there was no appreciable formation of ribulose. This raises the question as to whether the alfalfa leaf phosphoriboisomerase of Axelrod & Jang (37) does not perhaps also lead to the formation of xylulose. The assay method, based on a modification of the Dische-Borenfreund cysteine carbazole test, might not necessarily discriminate between ribulose and xylulose. The basis for excluding xylulose from the products has been that the dephosphorylated reaction products appeared to give only two spots on chromatography, coincident with ribose and ribulose. These responded in the expected way to the orcinol spray reagent of Klevestrand & Nordal (133).

In this connection one should note the observation of de la Haba and Srere [unpublished results cited by Racker (125)] that purified alfalfa leaf pentose isomerase transforms ribose-5-phosphate into a mixture of ketophosphates of which only a small portion is ribulose-5-phosphate. The susceptibility of only a small portion of the ketophosphate fraction to attack by crystalline transketolase is the basis for their belief.

The possibility that pentose phosphates other than ribose, ribulose, and xyulose may be involved in some of these isomerase reactions is raised by the observation of Dickens & Williamson (132) that approximately the same ratio of ketopentose phosphate to total pentose phosphate resulted with the rabbit muscle enzyme as with the horse erythrocyte enzyme. In the latter, the only components at equilibrium were ribose-5-phosphate and ribulose-5-phosphate; it should be impossible to have the same ketose to pentose ratio with the rabbit muscle preparation since a third component (ribulose phosphate) is involved, unless there were a compensating formation of a nonketo pentose or derivatives thereof.

The biological formation of L-xylulose has been known since Levene & LaForge (134) identified the pentose in the urine of pentosurics as this

sugar. Subsequently it has been shown by Touster, Hutcheson & Rice (135) that L-xylulose could sometimes be found in the urine of normal humans and of laboratory animals. Following the lead of Enklewitz & Lasker (136), these workers demonstrated that the administration of D-glucuronolactone increased the output of L-xylulose by normal as well as by pentosuric subjects. Here the pentose phosphate pathway acting in conjunction with the D-xylulose phosphate isomerase [found by Ashwell & Hickman (130) in mouse spleen] could not have been operative.

6-Phosphogluconate cleavage.—Entner & Doudoroff (137) pointed out a new pathway for the breakdown of glucose upon their discovery that *Pseudomonas saccharophila* converted this sugar to pyruvate and D-glyceraldehyde-3-phosphate. They were led by their experiences to suggest the following sequence of events (Fig. 3).

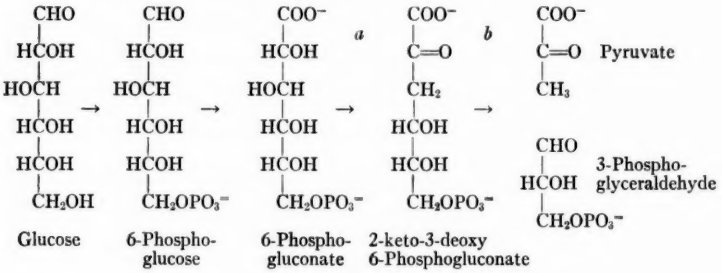


FIG. 3. The Entner-Doudoroff scheme.

The novel features of this scheme were the following: enzyme *a* (the phosphogluconic dehydrase), enzyme *b* (the "splitting enzyme"), and the hitherto unknown 2-keto-3-deoxy-6-phosphogluconic acid. Success by MacGee & Doudoroff (138) in the separation of these two enzymes from a cell-free extract and the isolation and identification of the 2-keto-3-deoxy compound verified this hypothesis. The same sequence was established by Kovachevich & Wood (139, 140) for *P. fluorescens*. No evidence has yet been brought forth that this mechanism occurs in higher plants. The obvious relationship of the keto compound to D-deoxyribose (a vital component of all plant cells) make it attractive to consider the possible place of this scheme in higher plants.

Incidentally, the operation of this scheme would introduce a new variable into the experiments in which the radioactivity of CO_2 liberated from specifically labeled glucose is used as a guide to ascertaining the pathway of glycolysis. It is obvious from inspection that CO_2 would arise preferentially from positions 1 and 4, although it is not necessary that both positions contribute equally since the two 3-carbon products are different. It is also obvious that recombination of triosephosphate to hexose would also obscure interpretation of labeling results.

Ascorbic acid formation.—The progress in the understanding of the biogenesis of ascorbic acid in plants owes much to the early work in animals. In tracer experiments with glucose Jackel *et al.* (141) established the likelihood that in the rat the carbon skeleton of glucose could be incorporated directly into ascorbic acid. Horowitz *et al.* (142) then showed that the carbon 1 of glucose became carbon 6 of ascorbic acid. Isherwood, Chen & Mapson (143) in a brilliant series of experiments based on the stereochemical relationship between the various hexoses and their corresponding onic and uronic acids found that the lactones shown below (Fig. 4) could give rise to ascorbic acid not only in the rat but also in the water cress seedling. Horowitz & King (144) also demonstrated the direct conversion of carbon-14 labeled glucuronolactone to ascorbic acid in the rat. Isherwood *et al.* (143) employed the lactones because of the poor penetration of the ionic forms of acids into plant tissue. In the case of galacturonic acid the methyl ester was used because the lactone does not form. Mapson & Isherwood (145) have recently succeeded

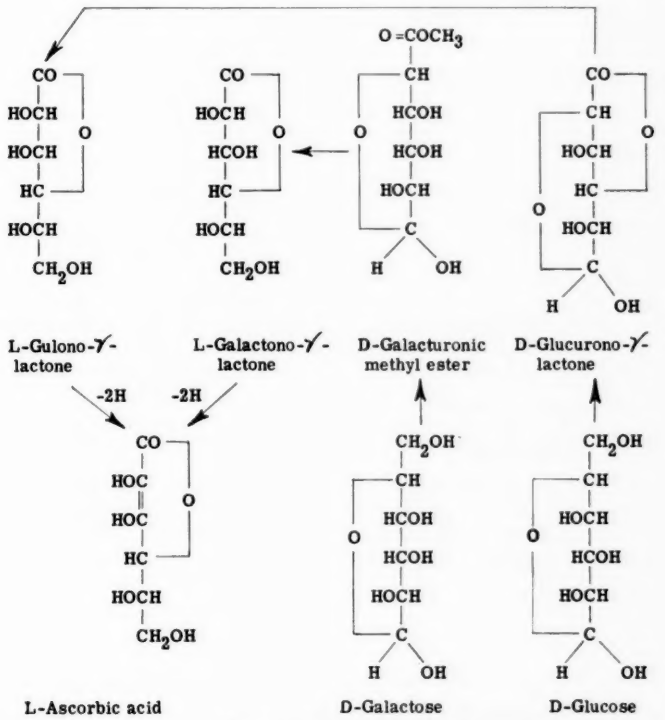
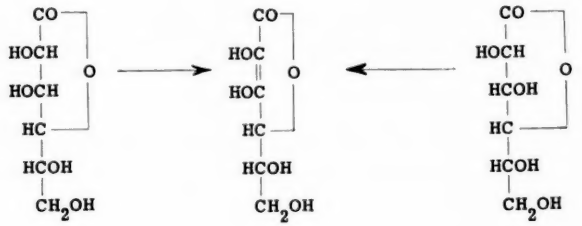


FIG. 4. Structural relationship of precursors to L-ascorbic acid.

in isolating the step: D-galacturonic acid→L-galactonic acid. A TPN-requiring enzyme is involved.

While the relationships shown appear to hold equally for both rat and water cress, the rat is unique as Isherwood *et al.* (146) have shown in converting D-mannono-γ-lactone to araboascorbic acid, while water cress alone has has the faculty of producing the same compound from D-altrono-γ-lactone (Fig. 5).



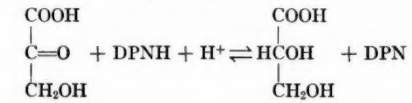
D-Mannono-γ-lactone D-Araboascorbic acid D-Altrono-γ-lactone

FIG. 5. Precursors of D-araboascorbic acid.

Mapson *et al.* (147) succeeded in converting L-galactonic-γ-lactone to ascorbic acid with mung bean and pea mitochondria from young plants. This remarkable achievement is the first instance of a cell-free biosynthesis of ascorbic acid. Oddly enough L-gulonolactone, which is an effective precursor in the intact water cress seedling, did not yield ascorbic acid. The plausible explanation is offered that this lactone needs first to be converted to L-galactonolactone by an enzyme not present in the mitochondrial preparations.

Isherwood *et al.* (148) also noted a parallel in the content of D-glyceric acid and L-ascorbic acid in water cress seedlings subjected to a variety of conditions. However the administration of L-gulonolactone or D-glucuronolactone led to an increase in ascorbic acid but to a decline in D-glyceric acid. These results, of course, weaken the argument that D-glyceric acid might be a precursor of ascorbic acid.

The discovery by Stafford *et al.* (149) of a D-glyceric dehydrogenase specific for DPN and distinct from lactic dehydrogenase is pertinent to the question of the origin of glyceric acid. The reaction shown below greatly favors the reduction of hydroxypyruvic acid.



Hydroxypyruvic acid D-Glyceric acid

It is tempting to speculate, in view of the results of Mapson *et al.* (147), that hydroxypyruvate might have its origin in an aldol-type cleavage of a keto-

hexonic acid. Isherwood *et al.* (143) did indeed consider the condensation reaction as a possible route to the ketohexonic acid, which, chemically at least, is a potential precursor of ascorbic acid.

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PHYSIOLOGY OF SEED GERMINATION[1]

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A seed contains an embryonic plant in an inactive condition, and germination is its resumption of growth. The young plant, protected by varying layers of living and dead tissues, has reserves for metabolism. In some seeds development starts as soon as water is absorbed, but in others germination does not take place until additional requirements are fulfilled. Three distinct stages are evident in germinating seeds, namely (*a*) imbibition of water, (*b*) cell elongation, and (*c*) increase in cell number. In a physiologic sense the start of germination depends upon coupling of respiration to growth. The established seedling results from resumption of development and its continuation through growth.

This review is restricted to consideration of processes involving resumption of development rather than the continuation of growth. The latter involves use of reserves for growth and the eventual development of photosynthesis and may be treated best as a separate subject. This restriction allows a single subject to be circumscribed, namely, the nature of the dormancy that blocks the initiation of growth. A statement of the present development of the subject is undertaken rather than a review of current work. Accordingly, references are required to some older literature, significance of which is now more evident, and references to the routine type of current observations are omitted.

Earlier work on seed germination was summarized by Lehmann & Aichele (98), Crocker (35), and Crocker & Barton (36). A succinct summary is given by Stiles (140). Recent interest in the physiology of germination has centered chiefly on the action of inhibitors and of light. Evenari (45, 47) has reviewed the work on these subjects prior to 1949 and 1952, respectively.

The Germination Process

What happens when germination starts? The first change when seeds are placed to germinate is the imbibition of water. In the dry seeds of maize (158) and of bean (109) all of the tissues are shrunken, cell vacuoles are small, the nucleus is irregular, and the cell contents are plasmolyzed, but with absorption of water the cells become turgid. During the first 10 to 12 hr. no elongation of cell walls can be noted nor have chemical changes been detected.

Elongation of cells of the coleorhiza of maize (125, 158) can be observed about 20 hr. after the beginning of imbibition. The coleorhiza breaks the peri-

[1] The survey of the literature pertaining to this review was concluded in September, 1955.

carp and extends about 2 mm. beyond the surface. The radicle elongates to fill the extending coleorhiza and soon breaks through the sheath which does not develop further. Cell division is first observed in the root tip at about the time it breaks through the coleorhiza. Cells of the scutellum enlarge greatly without dividing and the nuclei become very prominent. Soon after emergence of the coleorhiza (3) the coleoptile and plumule push through the fruit coat. In the germination of tobacco seed (4) the coat is broken by the developing primary root and the hypocotyledonary axis continues elongation. During this time the cotyledons remain within the seed coats. General observation shows that these sequences are the usual ones in the germination of seeds.

Enzymes may be assumed to be active early in the germination process. Horning & Petrie (76) in 1927 observed in the scutellum of dry seeds of maize, wheat, and barley numerous particles which they identified as mitochondria. They also found a few particles in the endosperm. With germination the mitochondria of the embryo increase in number and migrate to the nearby endosperm where they are associated with the corrosion of starch grains. Hageman & Hanson (63) recently segregated mitochondria from the scutellum of germinating maize seeds and found that enzymes associated with these particles hydrolyze maltose and soluble starch. Middendorf (109) observed mitochondria in the dry seeds of maize and bean and found that with the start of germination they elongated into rods. Radu (131) also observed an increase of mitochondrial elements in the epithelial cells of the scutellum of maize during germination but questioned the observation of Horning & Petri (76) that the mitochondria pass from the epithelial cells to the endosperm. Mitochondrial particles showing oxidative activity were extracted from four-day etiolated oat seedlings by Tager (150), from one-day seedlings of mung beans by Fritz & Naylor (57), from etiolated soybean seedling hypocotyls by Switzer, Smith & Loomis (149), and from peanut cotyledons by Kmetec & Newcomb (89). The extent of the mitochondrial activity is far from clear as the oxidative mitochondria of recent work were not tested for activity on carbohydrates and the particles of Horning & Petri were not tested for oxidative activity. One of the early phases of the germination process must be the activation of the mitochondrial-enzyme systems.

Material for the release of energy and for synthesis is required for cell elongation. Some sucrose is found uniformly distributed in the embryo of the dry grain of maize (158) where it may be the reserve substrate for respiration, but reducing sugar, as evidence of enzymic change, is first detected in the tip of the coleorhiza at the time it starts to elongate. Reducing sugar rapidly increases in the coleorhiza and radicle as these elongate. When the mesocotyl begins to elongate, reducing sugar first appears in this tissue. Accompanying or even preceding the appearance of reducing sugars, soluble peptides appear in the active regions of the embryo. Poljakoff-Mayber (127) indicated the presence of appreciable amounts of sucrose in dry seeds of

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Grand Rapids lettuce and the decrease of this sucrose in the first 24 hr. of germination. Stokes (143) showed that in the after-ripening of dormant seeds of *Heracleum sphondylium* the storage proteins are broken down into soluble compounds available for embryo growth. In light-sensitive seeds of *Digitalis purpurea*, Grohne (62) showed that no detectable changes beyond imbibition take place in the dark but within 3 hr. after exposure of the imbibed seeds to light, small grains of starch appear in the cells of the radicle. These grains increase in number and later appear in the endosperm cells near the root tip. Grohne stated that presumably the appearance of the starch grains is preceded by enzyme activity by which reserve fats of these cells are converted to sugar. Starch appears before elongation of the cells of the radicle, which does not occur until about 18 hr. after exposure of the seeds to light.

The requirement of energy for the onset of germination is satisfied by an increased rate of respiration. In seeds whose germination is blocked by a light requirement, Kipp (88) found that in the dark respiration increases with imbibition of water and then falls gradually after imbibition is completed. However, if the fully imbibed tobacco seeds are exposed to light, respiration again increases gradually. Hagen *et al.* (64) and Leopold & Guernsey (99) found that respiration of imbibed light-sensitive lettuce seeds held in the dark proceeds at an increased rate immediately after exposure to red radiation. After exposure to far-red radiation, the rate of respiration is slower. Using seeds of Grand Rapids lettuce, Evenari *et al.* (51) demonstrated an increase of respiration within an hour after promotion by red light. With ascospores of *Neurospora*, Sussman *et al.* (147) showed that, after treatment at a high temperature, the oxygen uptake and carbon dioxide evolution of the activated spores increase immediately.

To summarize, onset of the germination process after imbibition of water involves increased enzyme activity in the region of the radicle and an increased respiratory rate, followed by elongation of the radicle. In fact, one of the first measurable evidences of the onset of germination appears to be an increase of respiration. Neither the time sequence nor interrelationship of the changes has been established. The developing pattern of enzymatic activity in the seed has not been studied in a modern sense. This onset of germination is to be distinguished from subsequent growth and development of the seedling which are dependent on utilization of the main food reserves of the seed and the establishment of photosynthesis.

Factors Affecting Germination

Dormancies of seeds are biologically significant in spreading or delaying germination until the environment is favorable for development of the seedlings (7, 35). Certain conditions must universally be met for germination: moisture for rehydration, temperature within a suitable range, and oxygen for respiration. Seeds with other requirements for resumption of growth are of value in yielding information about the detailed steps in the germination

process. The combinations of factors necessary for germination of seeds have been studied widely as an applied science. The controlling mechanisms play an important role in the ecology of plants, persistence of weeds, and selection in plant breeding. The part played by environmental factors in determination of desert flora, for example, has excited much interest (6, 90, 175, 176, 178). These factors are also involved in the persistence of weed seeds in the soil (31, 38, 160) and in the distribution of weed seeds by irrigation water (25).

Desiccation of the seed as it matures is a conspicuous means of achieving a pause in the ontogeny of the plant. As a result of man's selection of seeds that germinate promptly, desiccation remains the main block to renewed growth in the seeds of many, but not all, crop plants. Seeds of most noncultivated plants almost universally have special blocks to germination as a result of natural selection. The blocks to germination that will be discussed are effects associated with covering layers, presence or absence of inhibitors or promoters, temperature requirements differing from those of growth, and light and dark. These blocks, or inhibitors, are eliminated under natural conditions by time, temperature changes, leaching, decay of surrounding fruit tissue, light, and other ways.

Coat effects.—Crocker (33) as early as 1906 was aware of most ways in which seed coats or other seed coverings are currently understood (35, 36) to affect germination. The effects are attributed chiefly to impermeability of the coats to water and dissolved gases, particularly oxygen and carbon dioxide, to the mechanical strength of the coats of some seeds which is believed to be sufficient to restrain the enlargement of the embryo, and to the occurrence in the coats of chemical inhibitors which are discussed in the following sections.

Breaking or removing seed coverings frequently improves germination. The limiting structure is often the seed coat proper, or it may arise from nucellar or endosperm tissues internal to the seed coat or it may consist of the pericarp and other external structures.

Germination of seeds of many grasses, for example, is improved by cutting, breaking, acid-treating, or removing certain seed coverings. Thus, Akamine (1) improved germination of several different species of Hawaiian range grasses by breaking the coats as did Johnson (83) with *Avena fatua*. Toole obtained similar results with *Danthonia spicata* (166), *Oryzopsis hymenoides* (167), and several species of *Sporobolus* (168). Brown *et al.* (16) found that barley and oat seeds that did not germinate at low temperatures if the seeds were intact germinated promptly at those temperatures if the coats were removed from over the embryos. In the grasses the seed coat is derived from both pericarp and remnants of the integument, and it is not known which part has an inhibitory influence.

Akamine attributed the beneficial effects of breaking the seed coats to improved gas exchange in eight species and to the release of mechanical restraint imposed on the embryos by the tough enveloping lemmas and paleas

in two species. Johnson was of the opinion that the coats of *Avena fatua* seed restricted oxygen entrance, and Toole also concluded that gas exchange was limited by the seed coats in the species with which she worked. In none of the grasses examined was the intake of water reported to be significantly inhibited by seed coats or other seed coverings.

Direct measurements of movement of oxygen, carbon dioxide, and other gases across seed coats were made by Brown (17). The inner seed coat of *Cucurbita pepo* is more permeable to gases than the outer, but it nevertheless functions as the rate-limiting layer because the outer membrane is perforated by the micropyle. Permeability of the inner coat is greatest when the membrane is slightly less than completely saturated with water. It contains a layer of living cells, the death of which increases its permeability. Many of the reports that the coats limit gas exchange are based largely on improved germination in atmospheres enriched with oxygen. Such evidence, however, does not necessarily prove that the limitation removed by the added oxygen was on rate of movement through the coats. It is generally assumed that increased oxygen availability accounts for the improved germination when the seed coats of nonhard seeds are opened. The act of opening the membrane may, however, through alteration of the embryo, be the factor promoting germination.

Germination of lettuce seeds does not occur at temperatures of about 30°C., but the embryos are able to grow at that temperature if all the seed coverings are removed. Borthwick & Robbins (14) studied the development of the three coverings that envelop the mature lettuce embryo. These coverings consist of an outer one derived from remnants of the pericarp, a middle one made up of the outer epidermis together with several layers of obliterated cells of the integument, and an innermost covering composed of two layers of endosperm to the outer surface of which adheres a suberized membrane, the outer wall of the inner integument. They found that germination at high temperature did not occur unless the innermost of the three membranes was removed. In this instance the inhibitory action may result from the integumentary portion of the innermost covering, but a possible direct influence of the endosperm is not excluded.

Evenari & Neumann (49) found that removal of coats from lettuce seeds eliminated their light requirement for germination, and Kugler (91), working with *Arabidopsis thaliana*, also found that the light requirement was lost if the coats were removed or even pricked. Evenari concluded that the innermost membrane of the lettuce seed was the effective one and that the beneficial effects observed when the pericarp alone was removed really resulted from incidental damage to the inner membrane. We, likewise, found with light-sensitive Grand Rapids lettuce seed that disturbing the external coat almost invariably rendered the seeds uncontrollable by light regardless of whether the coat removal was done in light or dark and with imbibed or dry seed. All coat structures, however, can be perforated with a needle without loss of subsequent controllability of germination by light if the seed is sup-

ported only from below and is not otherwise held when the needle is forced through the coats and into the cotyledon. From this, it appears that the visible damage to the seed coats is not so important as the way in which it is done. The mere flexing of the seed as it is held at one end while a portion of the pericarp is removed from the other may cause internal physical changes that result in loss of germination control by light without any actual perforation of the endosperm coat.

Impermeability of seeds to water is exhibited in a number of plant families but particularly by many species of Leguminosae in the form of so-called hard seeds. Because of the great agricultural significance of hard seeds, both of weeds and of cultivated crops, much research has been devoted to the development and structure of their impermeable coats, to the mechanism by which such coats allow water to leave but not reenter the seed, and to various conditions and procedures that will make such coats permeable without reducing the vitality and the agricultural value of the seed. This work has been evaluated from time to time by various reviewers, including Hamly (66), Zimmermann (183), Watson (174), Porter (130), Cavazza (30), and Hyde (77), and thus does not require further detailed review.

The work of Hyde merits further comment, however, because it gives a reasonable explanation of the mechanism by which hard seeds achieve and maintain a very low percentage of moisture despite wide fluctuations in the moisture content of the surrounding air. He found in mature seeds of *Trifolium repens*, *T. pratense*, and *Lupinus arboreus* that the hilum functioned as a ". . . . hygroscopically activated valve in the impermeable epidermis of the testa." In the early stages of ripening, water loss occurs freely through the epidermis, but, as the moisture content of the seed reaches about 25 per cent of the air-dry weight, loss through the epidermis begins to decrease, and below 14 per cent further loss of water is almost entirely through the hilum. The valvular action of the hilum results from a fissure in the epidermis along the groove of the hilum. This fissure was seen by Hyde to open in less than a minute when the seed was surrounded by dry air and to close equally rapidly in the presence of moist air. Anatomical features make closure of the fissure so positive that increase in weight of the seeds after many days in saturated air is not measurable. If the seeds are held at a humidity only slightly above that in equilibrium with the interior of the seed, however, the fissure in the hilum does not close completely, and the seed gradually absorbs moisture. This work explains and reconciles some of the seemingly contradictory observations of earlier workers and, in our opinion, constitutes an important contribution to an understanding of hard seed.

The possibility that the locus of light action is in the coats of light-requiring seeds has been studied through reciprocal crosses involving light-sensitive and light-insensitive parents. Honing (72) reported in 1926 that the need for light to promote germination of certain kinds of tobacco was a dominant character and that indifference to it was recessive. Since reciprocal crosses involving these two kinds always resulted in light-sensitive F_1

progenies, he concluded that the site of light action was not in the coat but in the embryo. In later experiments, Honing (73) found that reciprocal crosses did not always result in seeds that had exactly identical sensitivities to light. In general, however, he observed that either parent could effectively control the need for light but that the female frequently exercised a slightly greater influence than the male. He interpreted these results as evidence of joint cytoplasmic and chromosomal control.

Kugler (91), working with reciprocal crosses between light-requiring and light-indifferent strains of *Arabidopsis thaliana*, found the light-indifferent condition to be dominant, in contrast to the finding of Honing (72) for *Nicotiana* that the light-requiring condition was dominant. Kugler found variability between results of reciprocal crosses and she, like Honing, ascribed this to a maternal cytoplasmic influence. She found no evidence that the seed coats were directly involved in the action of light.

Conditions under which seeds are produced may influence their subsequent germinability, and in some instances the effects are correlated with structural differences in the coats. Lona (101), for example, found that seeds of *Chenopodium amaranticolor* were mostly dormant if the parent plant received long photoperiods during seed maturation but were readily germinable if matured on short days. The seeds matured under short-day conditions had appreciably thinner coats than those matured under long-day treatments. Although the dormant seeds germinated promptly if the coats were removed, the coats imposed practically no restraint on water uptake. Their hindrance to germination was regarded as a mechanical one.

Inhibitors and promoters.—Fugitive substances associated with the seed or fruit often cause seed dormancy. These substances have been considered in recent reviews by Evenari (45) and Moewus *et al.* (111). One of the first to be recognized was parasorbic acid, which was separated from fruits of *Sorbus aucuparia* by Hofmann in 1859 (71). It was shown by Kuhn & Jerchel (93) in 1943 to be the β-unsaturated lactone hexene-2-olid 1,5. Much of the physiological literature on inhibitors is concerned with the widely occurring β-unsaturated lactone coumarin which was shown as early as 1907 (136) markedly to inhibit root growth at concentrations of 7×10^{-6} *M*.

Evenari (45) listed the natural germination inhibitors that have been identified as ammonia, hydrogen cyanide, ethylene, essential oils including both aldehydes (citral, cinnamaldehyde, salicylaldehyde, benzaldehyde) and mustard oils (allyl- and β-phenethyl-isothiocyanates), alkaloids (cocaine, caffein, physostigmin, nicotine, etc.), unsaturated lactones (coumarin, protoanemonin, parasorbic acid), and unsaturated acids (cinnamic, caffeic, ferulic). To this list might be added dehydracetic acid and desacetyle (96) and phthalids. Phthalids were isolated by Naves (117) from essential oils of roots of *Levisticum officinale* and shown by Moewus & Schader (113) to be effective germination inhibitors of seed of *Lepidium sativum*.

Evenari (45) listed about 100 species of plants from which germination inhibitors have been isolated. In more than 60 of the species the inhibitor

was found in seeds or fruits, and in 22 species seeds were specifically mentioned as the source. Other examples continue to be noted (9). Randolph & Cox (133) found the endosperm of iris to contain substances that prevented germination. The naked embryo grew very readily but was inhibited by the presence of even a very small piece of endosperm. The inhibitory substance in seeds of *Cucurbita* was found to be present in both the seed coat and the embryo (85). The inhibitor was found in *Pennisetum ciliare* (1) to be in the bristles, glumes, lemmas, paleas, and other structures external to the seed but adherent to it. Germination inhibitors were found by Stout & Tolman (145) to occur in the extracts of beet seed balls. They ascribed the effect to the action of ammonia liberated by nitrogenous compounds in the pericarp and other structures of the seed ball exclusive of the seeds. De Kock *et al.* (40) consider that ammonia is secondary in its action to an unsaturated oil that can be extracted from beet balls. Shuck (138) obtained an inhibitory substance from lettuce seeds and Stout & Tolman (146) found that an extract from Grand Rapids lettuce seed was more inhibitory to the germination of beet seed than was a beet seed-ball extract itself. Kugler (92) extracted from the seed coat and embryo of seeds of *Sinapis alba* (*Brassica alba*) a fraction that fluoresced in ultraviolet light and that inhibited germination of *Sinapis* and other seeds. From chromatographic analysis it was assumed that amino acids and related compounds rather than oils were the important inhibitors. Germination inhibitors have thus been found in practically all of the structures that act as coverings of the seed or as coverings of the embryo within the seed. They have been abundantly reported in other plant parts not structurally associated with seeds but which come in contact with them in the soil after the seeds are shed (45, 181, 182).

Certain of the most effective germination inhibitors are illustrated in Figure 1. To this group might be added some of the salts of such heavy metals as copper and mercury.

Some of the inhibitors are rather specific for the germination process in contrast to the antecedent respiration or to many of the subsequent changes of the seedling. These specific inhibitors, moreover, all influence cell elongation of roots. This is perhaps best shown by the findings of Mayer & Evenari (107) that 26 derivatives of coumarin fall in closely the same order as inhibitors of radicle emergence of lettuce and as inhibitors of wheat root elongation. Coumarin was more effective on both processes than any of its derivatives. The parallelism of action was also observed for a group of phthalids and hexahydrophthalids on *Lepidium sativum* seed germination and root growth (113). The inhibitory action of coumarin on germination of lentil seeds was exceeded by that of (α-naphthyl)-3 hydroxy-4 benzo-6,7-coumarin, and several related compounds (95).

Coumarin and daphnetin (7,8-dihydroxy coumarin) were found by Burström (27) to inhibit the action of auxin in promoting the first phase of cell wall elongation which he considered was the result of plasticity arising from dissolution of the cell wall material. Coumarin and five of its derivatives

XUM

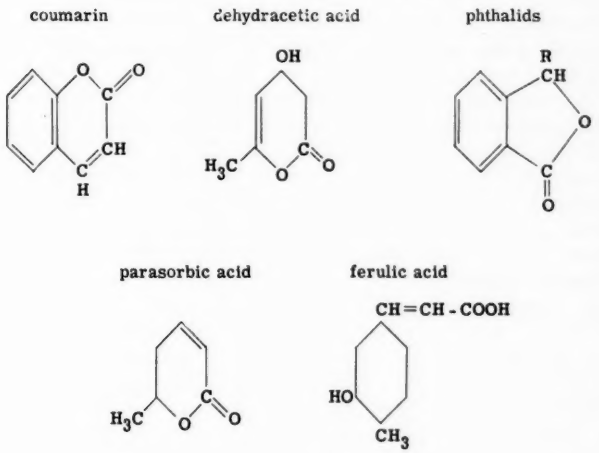

FIG. 1. Germination inhibitors.

were shown by D'Amato (37) to be "preprophase" inhibitors of mitosis. At high concentrations they upset spindle function.

Inhibitors applied at low concentrations generally promote germination of both seeds (45) and spores (110) as well as cell elongation (151). Coumarin promotes germination of *Chlamydomonas* zygotes at concentrations of about 10^{-7} *M* (110), which is about one one-hundredth that effective for germination of seed. The effectiveness of *cis*-cinnamic acid (promotion at 10^{-12} *M*) on the zygotes is most striking (110). A low effective concentration per se should not be questioned since the concentration refers to the ambient solution and not to the place of action. The maximum promotion caused by coumarin on lengthening of *Avena* coleoptile section (151) occurs at the same concentration (about 2×10^{-5} *M*) as for promotion of germination of *Lepidium virginicum* seed (164). This strongly supports the conclusion that radicle emergence is part of the same process expressed in elongation of cells in other plant tissues.

In 1922, Oppenheimer (123) recognized the similarity of the action of inhibitors on seed germination and on the germination of gemmae of *Marchantia* and spores of *Funaria*. The equivalence of the processes has been further supported by Moewus & Schader (113) not only for the growth of *Marchantia polymorpha* rhizoids [note also J. Rousseau (134)] but also for pollen germination and pollen tube growth of *Impatiens balsamina* and zygote germination of *Chlamydomonas eugametos*. Sprouting of buds of potato tubers is also inhibited by coumarin (112).

Seeds respond differently to these inhibitors depending upon whether, like lettuce, they are dormant and light-requiring, or nondormant like wheat or

maize (53, 106). In light-requiring seeds the inhibitor may be effective in preventing radicle emergence at concentrations of less than 10^{-4} *M*, while in nondormant seeds this condition is approached only at concentrations greater than 10^{-3} *M*, which really are toxic rather than inhibitory levels. Nutile (119) found that if nondormant lettuce seeds were imbibed in coumarin solutions and then thoroughly washed before the radicle emerged inhibition or root elongation was decreased. The dependence of the action of an inhibitor on its concentration is confused by possible destruction and the degree of penetration of the inhibitor into the seed. Mayer (105) found that coumarin disappears by enzymatic action in the presence of seeds.

Growth-regulating compounds such as indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid, and maleic hydrazide are relatively inactive as inhibitors of seed germination. Measurable effects are usually obtained only at concentrations greater than 10^{-3} *M*, while effects in growth tests are often maximal at 10^{-5} *M* or less. This is illustrated by the results of Everson & Dunham (53) for action of the triethanolamine salt of 2,4-dichlorophenoxyacetic acid on germination of beans, wheat, and other crop plants, as well as on a number of weeds including *Thlaspi arvense*, *Brassica kaber*, and *Abutilon theophrasti*. The compound was shown to penetrate through the water-permeable seed coats of beans and corn. Similar results were obtained on cotton with alkanolamine salt of dinitro-*ortho*-secondary butylphenol by Swanson *et al.* (148). Nétien & Briffaz (118) found that maleic hydrazide at concentrations of 10^{-7} to 10^{-3} *M* did not markedly inhibit germination of seeds of a number of crop plants. Evenari & Mayer (48) found a slight inhibitory action of indole-3-acetic acid on germination of lettuce seed. The argument might be held that the growth-regulating compounds externally applied are not reaching the tissue controlling the elongation associated with onset of germination, or that they are not particularly effective in controlling this process.

Inhibition and promotion of germination or elongation are symmetrical with respect to their actions. Response as a consequence of direct promotion cannot immediately be differentiated from response attributable to poisoning of an inhibitor. Two types of promoter have been widely recognized, namely, nitrates and materials having an —SH function as cysteine, glutathione, and possibly thiourea.

Thiourea is known to be particularly effective in promoting germination of some light-requiring or temperature-inhibited seeds such as lettuce (152), and kok- and tau-saghyz (129). In fact, the light requirement is sometimes removed (132). Thiourea is used at very high concentrations (about 0.5 per cent) and severely inhibits subsequent growth unless removed after inhibition (152). The presence of a C=S group in thiourea with possible isomerism with C—SH strongly suggests that thiourea might be functionally related to glutathione and cysteine, which are effective at low concentrations.

An inhibitor of germination and elongation found in seed coats of Victory oats by Elliott & Leopold (44) was reduced in its action by 1,2-dithiopro-

panol (BAL) and glutathione. These two —SH-containing compounds were also found similarly effective on reversing the inhibition of *Avena*-section lengthening by coumarin and protoanemonin (151). Thimann & Bonner (151) suggested that this reversal of the action of β-unsaturated lactones by effective —SH-type compounds is associtaed with addition of the thiol group to the double bond as observed by Cavallito & Haskell (29) for the reaction of cysteine with Δ,α,β- and Δ,β,γ-angelicalactones. This interpretation was questioned by Mayer & Evenari (107), who pointed out that Cavillito & Haskell did not get a reaction between coumarin and dehydracetic acid or cysteine. In actuality the —SH group that is effectively inhibited might be part of an enzyme rather than of glutathione or cysteine, or the reaction with the inhibitor at the site of action might be enzymatically controlled. Elliott & Leopold (44) found that the inhibitor in *Avena* was also an inhibitor of alpha- and beta-amylase activity, which are generally inhibited by —SH antagonistic compounds. It also inhibits the effect of indoleacetic acid on pea stem elongation. This type of enzymatic inhibition was most strikingly evident in the action of 2,4-D on lipase activity (65, 94). Lipase production in lettuce seeds was also inhibited by coumarin during a 30-hr. germination period (128). Tietz (153) found indications of photoactivation in a lipase preparation from *Oenothera biennis* and noted that germination was accompanied by a decrease in the total fat content and an increase in its acid number.

The diffusible factor from roots of some plants that stimulates germination of seeds of *Striga hermonthica* and *Orobanche minor*, plants which parasitize grass and legume species respectively (18, 19), has been greatly concentrated by Brown and his coworkers (20, 21, 22, 24). Seeds of both plants are light-sensitive in part, those of *Striga* being suppressed in germination by light (24). The active factor from roots is a resinous material that can be evaporated at very low pressures. It is acidic in nature and is not a carbohydrate or amino acid. The same general type of isolated material is effective on *Striga* and *Orobanche*. The concentration range over which the material is effective is of the order of 1 p.p.m. and is rather narrow. In the work on *Striga* germination the activity of many sugars was tested (23). A material, separated from linseed, effective at the rate of a few million molecules per seed (*circa* 10^{-10} *M*) of *Orobanche*, was found to have the empirical formula C_{10-11} H_{16-18} O_4. The properties of the material suggested that it was a lactone with conjugated unsaturation, one possibility being the dilactone of a polyhydroxy, aliphatic, dicarboxylic acid. Some lots of D-xylulose preparations were found to be effective at concentrations as low as 10^{-7} *M*. This activity was fugitive, and, while preparations varied greatly in activity, strong evidence was given that the activity was attributable to D-xylulose. An interpretation that can be made is that one of the isomers involved in mutarotation is the effective compound, possibly an anhydride (102).

Nitrates were shown by Gassner (59) in 1915 to be effective promoters of

germination of light-sensitive seeds. Hesse (70) found that the dark germination of many, but not of all, light-sensitive seeds was increased by the use of solutions of KNO_3 to moisten the substratum. The greatest response was at 10^{-2} *M* with only slightly lower values at twice or half this concentration. Schwendiman & Shands (137) reported that a 0.2 per cent solution of potassium nitrate was equally as effective as prechilling in overcoming delayed germination of freshly-harvested seeds of Vicland oats. The action of nitrate solution varies with the temperature of germination (163). The germination of seeds of *Digitaria ischaemum* is promoted by nitrate solution at low temperature alternations that are unfavorable for germination but is inhibited at higher temperature alternations that are the most favorable for germination. Nitrates and nitrites both were found to be effective promoters of germination of seeds of four varieties of tobacco (120). Ammonium nitrate was considerably more effective than were other nitrates.

Hydroxylamine strongly promoted germination of tobacco seeds (120) at 0.001 *M* but was inhibitory in action at 0.005 *M*. Some response was obtained with amino acids, alanine and glutamic acids being particularly effective. Citric, α-ketoglutaric, fumaric, and malic acids, compounds of the citric acid cycle, all gave some promotion of germination. This was also true for malonic acid, which is generally considered to compete with succinic acid.

Temperature.—Many papers have been published on the "cardinal" germination temperatures, on the best constantly-maintained or alternated temperatures, and on the proper prechilling treatment for the germination of seeds of individual species. While few contributions have been made toward understanding the effects of temperature, the great variability of the temperature requirement among different species and within a species is established, depending on age, storage conditions, and other factors. In particular, an appreciation has been developed of the influences on germination of alternating temperatures, of time after harvest or maturity, and of storage conditions. Went (177) reviewed many of these general responses to temperature conditions.

Minimal, optimal, and maximal temperatures for germination were defined by Sachs (135) in 1860. Early work on the relation of temperature to germination was reviewed by Edwards (43). The conception of cardinal temperatures is fuzzy and observations on these temperatures, particularly on the minimal values, are sometimes confused with rate of germination. Nevertheless, a temperature region most favorable for germination does exist for particular lots of seed.

Freshly harvested seeds of some cereals were early found to have a comparatively low maximal temperature of 10° to 15°C. (2). As the seeds aged over several weeks, this maximal value was raised by about 10°C. The seeds of many plants show a sharp inhibition of germination with increase of temperature. Seeds of *Brassica juncea* (162), when tested immediately after

XUM

harvest, showed 97 per cent germination at 10°, 97 per cent at 15°, 63 per cent at 20°, and 8 per cent at 25°C. Three weeks later, germination was 95 per cent at 25°C. Fresh seeds of subterranean clover (161) also germinated best at low temperatures, but with this species the sensitivity to high temperatures persisted over a period of at least three months. On the other hand, some other freshly harvested seeds have an initially high minimum temperature that changes with aging. Freshly harvested seeds of *Alysicarpus vaginalis* (42) germinated best at 35°C., but after several months germinated equally well at lower temperatures.

Effects of daily alternations of temperature have been studied in relation to seed germination. In connection with an early report by Caspary (28) in 1860, and the studies of Stebler (139) in 1881, of the benefits of light in promoting seed germination, von Liebenberg (171) and Pickholz (124) pointed out that at least a part of this benefit was a result of the daily warming of the seeds by light. This was the beginning of extensive studies to establish the effective temperature range for daily alternations. The works of Harrington (67) and of Morinaga (114) are illustrative. Generally, an alternation from a temperature near the optimal constant value to some higher value which might be above the maximal is most effective. The practice is to hold the former temperature for about 16 hr. and the latter for 8 hr. as suggested by the day to night variation of surface soil temperatures in temperate zones (15).

Repeated cycles are required for many seeds including *Poa pratensis*. A single change of temperature from 15° to 25°C. in connection with exposure to light suffices to increase greatly the number of seeds of *Lepidium virginicum* (164) that will germinate. Germination of seeds of *L. virginicum* (165) at 20°C. in response to red light is greatly increased if the seeds are held at 35°C. for 2 hr. The suggestion is advanced that the increased respiration and metabolism at the high temperature change the balance of the intermediate materials of the respiratory cycle. This new balance may not be favorable for germination at a continued high temperature, but may promote germination with a change to a lower temperature. With seeds that require daily alternation of temperatures, more than one change of temperature may be required to attain a favorable balance of the various steps that lead to germination.

The need for temperature alternations as well as the most effective range of temperature varies with seed maturity at time of harvest and with the age of the seed (86). Conditions of storage influence the change of response to temperature as the seed ages. Seeds of *Festuca capillata* (86) do not germinate at 30°C. when freshly harvested, but after storage at 20° or 30°C. for a year the seeds have after-ripened and will then germinate fairly well at 30°C. Similar seeds stored at −10° or 2°C. still showed low maximal temperature requirements. A similar condition was noted by Brown *et al.* (16) for seeds of cereals.

The seeds of many temperate-zone plants require a long period under moist conditions at low temperatures for germination. This is the basis of the ancient horticultural practice of stratification. Apple seeds must be held moist at a low temperature (4°C.) for about two months before complete germination is possible. Luckwill (103) found that growth-promoting substances appeared in the embryos of after-ripened seeds just before germination started, but did not appear in seeds held in dry storage. He considered the growth-promoting substances to be more important in the control of germination than the inhibitors present in the dry seed. Vallance (169) worked with seeds of *Rhinanthus crista-galli*, which required from two to three months stratification before germination was possible, and noted an increase of respiration that started before after-ripening was completed. Also, the number of amino acids in alcohol extracts of the seeds increased during after-ripening and reached a maximum just before germination was apparent. Flemion (54) found that during after-ripening of moist rosaceous seeds at 5°C. increases occur in water-absorbing power, acidity, enzyme activity, and soluble materials including amino acids.

In some seeds, after-ripening changes which are morphologically evident take place at low temperatures. Stokes (141) studied the details of after-ripening of the seeds of *Heracleum sphondylium*, which at harvest have rudimentary embryos. When the seeds were held moist at 2°C., the embryos developed at the expense of the endosperm. After about nine weeks at the low temperature, germination started at 2°C. or continued at an increased rate at 15°C. or higher. When moist seeds were held at 15° to 20°C. the embryo started development but increased very little in dry weight and very soon stopped all development. Stokes (142, 143) associated the development at low temperatures with evidence of enzymatic action indicated by increase of soluble nitrogen compounds and an increased proportion of arginine (144) among the amino acids.

From a comparative point of view, a striking similarity is to be emphasized between these temperature responses and those evident in the breaking of bud dormancy. Parallels also are evident with diapause phenomena in insects. An attempt to correlate these facts with the interaction of temperature and light effects will be made after responses to light are considered.

Light and dark.—The subject of the role of light and dark in seed germination was discussed in a review completed in 1951 by Evenari (47). This and earlier reviews by Crocker (34, 35) describe most of the observed phenomena. Quantitative aspects of the subject have been developed since 1951. Promotion of seed germination by visible radiation was noted as early as 1860 (28), and inhibition of seed germination of *Acanthostachys strobilacea* was reported in 1903 (68). Both the inhibition and the enhancement of germination by light were widely confirmed, and numerous examples of each have been discovered. The action spectra for the responses were qualitatively known by 1940 (47). The responsiveness of some seeds and the indif-

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ference of others were evident (87), even though unappreciated. Flint & McAlister (56) in 1937 and Evenari (47) in 1951 recorded hunches that the photoresponses were similar to those of photoperiodic control of flowering.

That the photoreaction controlling germination of some lettuce seeds was repeatedly photoreversible on the same seed was established in 1952 (12) as was also the equivalence of the action spectrum in both directions with that controlling photoperiodic response of *Xanthium pennsylvanicum* (11). Identity of the controlling photoreaction has now been established by the specific photoreversibility for the following phenomena: germination of seeds of *Lactuca sativa* (12, 13), *Lepidium virginicum* (164), *Lepidium densiflorum* (164), *Nicotiana tabacum* (159), and *Lamium amplexicaule* (84); floral initiation of *Xanthium saccharatum* (11), *Glycine max* var. Biloxi (41), *Hyoscyamus niger* (41), and *Hordeum vulgare* var. Wintex (41); opening of hypocotyl hook of *Phaseolus vulgaris* (179); seedling etiolation of *Phaseolus vulgaris*, flax, peanut, and *Hordeum vulgare* var. Colsess (41); expansion of etiolated bean discs (100); cuticle coloration of *Lycopersicon esculentum* (126); and germination of fern spores (26). Some other responses probably subject to the same controlling reaction as suggested (69) by the knowledge about them, even though they have not been tested for photoreversibility, are leaf enlargement, epinasty, leaf abscission, bulb formation, rhizome production, Casparian strip formation, flower development, plant pigmentation, phylloidy of bracts, succulency, sex expression, response to day and night temperatures, and bud dormancies. The reversible photoreaction regulating seed germination is a general one of growth control. This generality permits comparative treatments of seed germination and the other responses.

Action spectra for the reversible promotion and inhibition of germination of *Lactuca sativa* and *Lepidium virginicum* seeds (164) are shown in Figure 2. The relative radiant energies required to produce a given response of light-sensitive seeds, such as 50 per cent germination, by either promotion or inhibition, vary with time of imbibition (13, 46, 50, 78, 121, 154, 164), temperature conditions during germination (46, 154, 159, 165), prior exposure to radiation (165), time and conditions of storage, and other factors. The sensitivity of the reversible photoreaction evidently depends upon the conditions to which the seeds are exposed prior to or during germination. Whenever the energy requirement for promotion of germination to a particular value varies, that for inhibition changes in the opposite direction (13, 159, 164). The several action spectra are closely similar on a relative energy scale despite considerations that might be raised as to the scattering or absorption of radiation before it reaches the receptive region of the seed.

Recent qualitative results on action spectra obtained with light filters are in agreement with the more quantitative work. These include germination of edible burdock seeds, *Arctium lappa* (115), and seeds of a number of other vegetables and crop plants (116). If imbibed light-sensitive seeds are exposed to unfiltered radiation from the sun or incandescent filaments, the

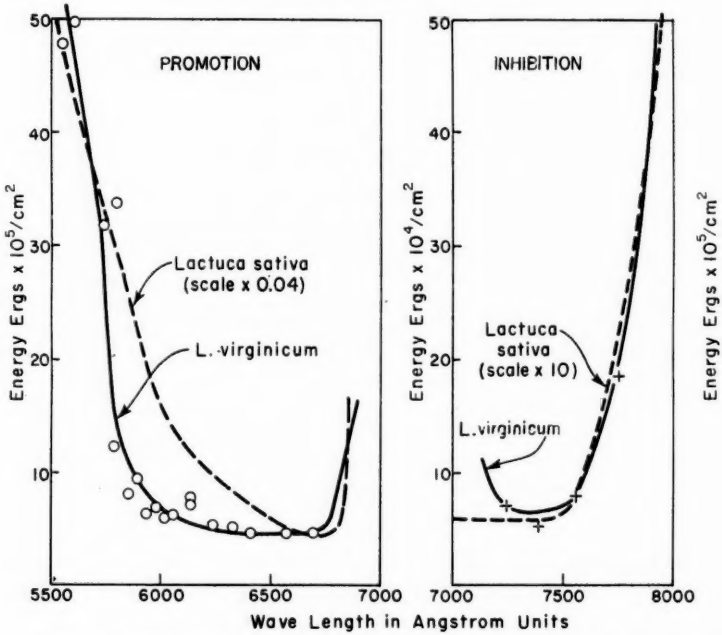

FIG. 2. Action spectra for promotion and inhibition to 50 per cent germination of *Lepidium virginicum* seeds at 20°C. in 0.2 per cent KNO_3 solution (solid lines). Action spectra for promotion and inhibition of germination of seeds of *Lactuca sativa*, shown for comparison (164, Fig. 2).

germination of some is promoted and of others inhibited. Examples of promotion (116) are mitsuba, celery, *Perilla*, and ramie, and of inhibition are watermelon, squash, cucumber, calabash gourd, balsam pear, eggplant, tomato, and pepper. The type of response depends upon the temperature and probably is determined by the relative sensitivities of the red (promoting) and far-red (inhibiting) photoreactions. The germination of all light-responsive seeds that have been tested are promoted by red radiation and inhibited by far-red radiation irrespective of their response to sunlight or radiation from incandescent filaments (47, 84, 108). Sensitivity to radiation, however, can change during the course of exposure (165).

The necessity of irradiation for germination of some seeds and the indifference of others are striking in many lots of seeds which might be studied as light-requiring but which still show some germination in darkness. This is of the greatest significance for an understanding of the series of controlling

reactions. Nutile (119), in fact, induced light sensitivity in non-light-requiring lettuce seeds by the action of coumarin. On a comparative basis the equivalent phenomenon in flowering is the night-length requirement of some plants and the indifference of others (69).

An important characteristic of the photoreaction, basic to the light response, is its photoreversibility (12). It can be written as follows:

$$\underset{\text{(Red absorbing)}}{\text{Pigment} + \text{Reactant}} \underset{\text{(Far-red: Peak } \textit{circa.}\text{ 7350 Å) (darkness)}}{\overset{\text{(Red: Peak } \textit{circa.}\text{ 6550 Å)}}{\rightleftharpoons}} \underset{\text{(Far-red absorbing)}}{\text{Pigment} + \text{Changed Reactant}}$$

[Note (52), however, for disagreement before the fact was established.]

Knowledge about the details of this reaction is undergoing rapid development and features are known that may not appear in print for a few years. One feature can be developed here, since it depends only upon published results. This is the calculation of the fractional conversion of the pigment required for a given percentage germination of responsive seed. The method follows that used by Warburg & Negelein (172, 173) in their study of the photoreversible inhibition of cytochrome oxidase by carbon monoxide. The calculations depend upon the fact that the photoreaction is first order in both directions as shown by a temperature coefficient of unity (13), and make use only of the variation of the germination with increasing radiant energy as have been published for *Lactuca sativa* (13) and *Lepidium virginicum* (164) seeds. Seeds of *Lactuca sativa* require 50 per cent conversion of the pigment to the far-red-absorbing form for 50 per cent germination, while those of *Lepidium virginicum* require only 17 per cent conversion for 50 per cent germination.

The lines expressing the probit for germination of *Lepidium virginicum* as a function of incident radiation energy in the red part of the spectrum are displaced parallel to one another as conditions for germination are varied (13, 164). This indicates that the incident energy required for a given fractional conversion of the pigment varies with conditions but that the corresponding per cent germination is constant. It is likely that such a result arises from variation of the quantum efficiency for pigment conversion which indicates that another non-rate-limiting reactant is involved in the photoreaction.

The pigments have not been detected by nonbiological methods. They have no prominent maxima of action in wave length regions greater than 4000 Å other than in the red for the one and the far red for the other (13). For each pigment the product of (molecular absorption coefficient) × (quantum efficiency for conversion) × (fraction of the incident light reaching the site of the photoreaction) is equal to the (fraction of the pigment converted) ÷ 1000 × (the incident energy as known from the action spectra). These values on the right side of the equation when expressed in Einsteins/cm.^{2} vary from the order of 10^3 to 10^5 (gram molecules/l.)$^{-1}$ for both forms

of the pigment depending on conditions for germination. Variations in germination conditions result in changed quantum efficiencies for conversion. The molecular absorption coefficients at the maxima of action are accordingly greater than 10^5, since the probable maximum value of the quantum efficiency is unity and the fraction of the incident light reaching the site of the photoreaction is less than unity.

Light and temperature interactions.—Many seeds have long been known to germinate in darkness at one temperature, usually near the optimum, but to require light for germination as the temperature is increased toward the maximum (5, 58). Some seeds that require light for germination at constant temperature will germinate in darkness under alternating temperature (60, 171) or in the presence of nitrate (59). The circumventing of a light requirement for germination is evidence that the biochemical changes brought about by the photoreaction can be attained through other reactions (165).

The photoreaction is not dependent on the temperature during the light treatment (13), but the temperature during imbibition in the dark and following the light exposure has a marked influence on the germination response (47, 55, 165). Lettuce seeds that ordinarily germinate equally well in light or dark become light-sensitive if the imbibed seeds are held several days in the dark at from 29° to 35°C. (13, 50). Seeds of *Arabidopsis thaliana* (91) require light for germination. Freshly harvested seeds require an alternating temperature, but fully after-ripened seeds germinate equally well at constant temperatures.

Seeds of *Lepidium virginicum* (164) germinate only after exposure to light. At constant temperatures of either 15° or 25°C. germination was never complete even with saturating radiation, but a single shift of the temperature from 15° to 25°C. at or near the time of exposure to light greatly increased the percentage germination.

Also holding the imbibed seeds at 35°C. for 2 hr. (165) markedly improved germination of light-promoted seeds of *L. virginicum* held otherwise at 20°C. If the period at 35°C. followed the light treatment, eight times the energy was required for promotion of germination to 50 per cent compared to the energy needed if the period at 35°C. preceded the light treatment. Holding the moist seeds at a high temperature for a short time changed the light sensitivity of the seeds.

Photoperiodic Response

Light-responsive seeds may vary in their germination depending upon the durations of light and dark in a pattern somewhat suggestive of photoperiodic control of flowering and other plant growth responses. Seeds of *Lepidium virginicum*, *Nigella damascena*, *Veronica persica*, and *Silene armeria*, as well as a number of other light-stimulated seeds germinated well with daily irradiations of a few minutes or hours, but poorly when held

continuously in either light or dark. They thus have features of short-day plants. *Leptandra sibirica* (79) and *Spiraea japonica* (82) responded most favorably to continuous irradiation, as also did seeds of birch, *Betula pubescens* (8). The latter, therefore, correspond to long-day types. Seeds of *Epilobium cephalostigma* required a short exposure to light followed by a dark period of six or more hours and a second light period of high total energy (80). The reaction involved in the second light period is possibly different from that in the first. The high energy requirement may be indicative of a response to the blue part of the spectrum or possibly to heating of the sample.

Germination of fern spores (81) was also found to depend upon the light period, being favored by long days, but showing differences depending upon light intensity. Spores of *Dryopteris crassirhizoma* germinated best at low intensity, while those of *Pyrrosia lingua* and *Microsorium ensatum* responded best to high intensity. Spores of *Asplenium prolongatum* were indifferent to variations in light intensity from 10 to 1000 lux.

Glutathione and Ascorbic Acid Relationships

An argument was early advanced that radiation influenced germination of light-sensitive seeds by displacement of an oxidation-reduction system toward the reduced state (10, 88). Glutathione (GSSG⇌GSH) and ascorbic acid (AA⇌DHA) have been suggested as components of this system since they often appear at about the same time in the course of germination. Hopkins & Morgan (75) found that the concentration of glutathione reached a maximum value in the early stages of pea seed germination. This finding was questioned later (97), a marked increase in the ascorbic acid being noted instead. The difficulty is in the specificity of the analytical methods. The system

$$2\,GSH + DHA \rightleftharpoons AA + GSSG$$

$$AA + \tfrac{1}{2}O_2 \rightleftharpoons DHA + H_2O$$

was considered as a possible hydrogen-transfer system by Mapson & Goddard (104). For this system to be functional, GSSG must be reduced at an adequate rate. They extracted from peas a glutathione reductase effective in the transfer of hydrogen from reduced Coenzyme II to GSSG. This enzyme was also found in wheat germ by Conn & Vennesland (32). Mapson & Goddard (104) also showed coupling of the system to triphosphopyridine nucleotide dehydrogenases. Malic enzyme, isocitric dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase form such coupled systems (170).

Reduced glutathione (GSH) can be nonenzymatically oxidized by dehydro-ascorbic acid (DHA). Plants, however, probably maintain ascorbic acid in the reduced state by the enzymatic interaction of AA and GSH controlled by dehydro-ascorbic acid reductase (170). The existence of this en-

zyme in cabbage and cauliflower juices was suggested by Hopkins & Morgan (74) in 1936.

Zinc, copper, lead, mercury, and cadmium ions at concentrations of *circa* 10^{-5} *M* cause 50 per cent inhibition of glutathione reductase (170). They are also effective in this concentration range as inhibitors of germination. The effectiveness suggests action through inhibition of free —SH groups of the enzyme (170).

Germinating seeds of *Vigna sesquipedalis* showed marked changes in their dehydrogenase patterns. Citric dehydrogenase activity was completely absent in the cotyledons of the ungerminated seeds, but appeared soon after germination started (122). Formic dehydrogenase, on the other hand, decreased with germination (39, 122, 180).

The ascorbic acid content of seeds is usually very low (155) but increases rapidly as germination proceeds (61). Tonzig (156) advanced the thesis that ascorbic acid raises the structural resistance of the cell wall to distortion; it opposes the action of auxin in this regard (157) as also does coumarin (27).

Résumé

Some conclusions about the germination process can be drawn in a general form. The process is controlled by a system of interlocking reactions that must be kept in proper balance. The balance required is so exact as to be attained only within narrow temperature limits with some seeds. If the seeds require only imbibition of water for germination, the system of reactions is complete and balanced. This implies the presence of readily available reserves within the developing radicle or its contiguous cells, the utilization of which is mediated by pre-existing enzymes. The first visible step of germination is cellular elongation, the control of which is still obscure even in regard to the energy requirement. Synthesis of enzymes for the utilization of the main seed reserves of the endosperm or cotyledons is not essential to these early steps, but follows closely. A process antecedent to the use of the major food reserves for seedling growth is emphasized.

Seeds requiring irradiation in the red part of the spectrum have a specific block for the first step of germination. Light-requiring and dark-germinating seeds are alike in their dependence on the process affected by the block, as is shown by the fact that it is possible to convert seeds of either type to the other type. The same process is involved in control of flowering, stem elongation, and many other growth responses mediated by light. This identity is shown by the reversibility of the photoreaction and the close similarity of the action spectra.

Coumarin and other compounds of a β-unsaturated lactone type act as specific poisons of the reactions associated with the controlling reversible photoreaction. This is known from the similarity of the action of these compounds upon the several related responses. The inhibitory action can be

reversed by glutathione, protoanemonin, and other —SH functional compounds.

Mitochondrial activity and enhanced respiration are noted early in the process of germination, but their exact relations to the initial step of radicle elongation are unknown. The dynamics of enzyme development are unknown for the greater part but lipase activity appears at an early stage in some seeds.

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PHOSPHORUS METABOLISM AND PHOSPHOTOSYNTHESIS[1,2]

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Introduction

The present article, being the first in the *Annual Review of Plant Physiology* devoted specifically to a survey of phosphorus metabolism in photosynthesis, will attempt to trace the conceptual and experimental evolution of this subject without adhering to chronological boundaries. Some of the papers discussed here at considerable length have already been reviewed, though necessarily less extensively, and often from a different point of view, in previous articles dealing with broader phases of photosynthesis (1, 2, 3). Not all aspects of phosphorus metabolism in photosynthesis will be reviewed; those pertaining to nutrition and general metabolism have been recently surveyed by Pirson (4).

Although phosphorylated compounds, including sugars, have long been known to occur in green plants (5, 6) and some early attempts were made to correlate photosynthetic CO_2 fixation with phosphorus metabolism (7, 8), the role of this element in photosynthesis has become a subject for systematic theoretical and experimental inquiry only in the last 12 years. The interest in this subject was awakened by the recognition of the key role played by phosphorus compounds, especially those of adenylic acid, in the release and transfer of chemical energy in the metabolism of heterotrophic organisms. The classical reviews by Lipmann (9) and Kalckar (10) were effective in stimulating interest in the question whether phosphorus compounds were also concerned in energy transformations during photosynthesis.

Early Hypotheses

Energy-rich phosphate as part of "assimilatory power."—The first comprehensive hypothesis on the role of phosphorus in photosynthesis was formulated by Ruben in 1943 (11). Ruben sought a mechanism for the photosynthetic assimilation of carbon dioxide consistent with a conclusion he reached in his pioneering studies with radiocarbon, i.e., that CO_2 fixation involves two dark processes: an initial incorporation of carbon dioxide into a carboxyl group of a suitable acceptor (RH) and the subsequent reduction of the carboxylated acceptor ($R \cdot COOH$) to the level of carbohydrate. Both of these reactions would be endergonic, and Ruben suggested that they were

[1] The survey of the literature pertaining to this review was concluded in August, 1955.

[2] The following abbreviations will be used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine-5-monophosphate; P_i, orthophosphate; TCA, trichloracetic acid. FMN, flavin mononucleotide.

wholly or in part carried out at the expense of the pyrophosphate bond energy of ATP.

He estimated the free energy change in the carboxylation reaction to be no greater than the energy released by the hydrolysis of one energy-rich phosphate bond of ATP. The exact chemical nature of RH was not certain, but he supposed that it was an aldehyde. He assumed that the CO_2 acceptor underwent a phosphorylation by ATP prior to the carboxylation step. Ruben represented the carboxylation process by equations 1 and 2:

$$\text{Energy-rich phosphate donor} + \text{RH} \overset{\text{Enzyme}}{\rightleftharpoons} \text{"free" donor} + \text{phosphorylated RH} \quad 1.$$

$$\text{Phosphorylated RH} + \text{CO}_2 \overset{\text{Enzyme}}{\rightleftharpoons} \text{RCOOH} + \text{inorganic phosphate} \quad 2.$$

The second phase of carbon dioxide assimilation in photosynthesis, the reduction of the carboxylated compound (R·CO·COOH) to a carbohydrate (R·CHOH·CHO), Ruben (11) visualized as involving a series of three reactions consisting, first, of a reduction (equation 3) of the carboxyl group by a reducing agent (which he thought was likely to be a pyridine nucleotide), followed by a phosphorylation of the carboxyl group (equation 4), and finally by the reduction of the phosphorylated carboxyl to carbonyl group (equation 5).

$$\text{R}\cdot\text{C}(=\text{O})\text{COOH} + \text{PNH}_2 \rightleftharpoons \text{R}\cdot\text{CHOH}\cdot\text{COOH} + \text{PN} \quad 3.$$

$$\text{R}\cdot\text{CHOH}\cdot\text{COOH} + \text{ATP} \rightleftharpoons \text{R}\cdot\text{CHOH}\cdot\text{C}(=\text{O})\text{OPO}_3\text{H}_2 + \text{ADP} \quad 4.$$

$$\text{R}\cdot\text{CHOH}\cdot\text{C}(=\text{O})\text{OPO}_3\text{H}_2 + \text{PNH}_2 \rightleftharpoons \text{R}\cdot\text{CHOH}\cdot\text{CHO} + \text{PN} + \text{H}_3\text{PO}_4 \quad 5.$$

Ruben reasoned that reduced pyridine nucleotide (PNH_2), with a redox potential of *circa* −0.3 volts at pH 7, was capable of reducing a carbonyl to a secondary alcohol, a reaction requiring *circa* 0.2 volt (equation 3), but not a carboxyl to a carbonyl group, a step requiring *circa* 0.5 volt (equation 5). The reduction of a carboxyl group by PNH_2 would be possible, however, after the potential barrier was first lowered by a phosphorylation of the carboxyl group (9) by ATP (equation 4).

In Ruben's scheme (11) the photochemical reactions of photosynthesis made CO_2 fixation in the dark possible, not by generating a single reductant with a reducing power sufficiently great to reduce carbon dioxide to the level of carbohydrate, but rather by generating two agents: a reductant with an intermediary reducing potential, approximately that of reduced pyridine nucleotide (PNH_2) and an energy-rich phosphorus compound ex-

emplified by ATP. The energy of ATP would be used for the carboxylation reaction (equations 1 and 2) and to assist in the thermodynamically difficult step of reducing a carboxyl group (equations 4 and 5).

The generation of these two components of what will be called in this review the assimilatory power was linked in Ruben's view with the photolysis of water, as represented by equation 6.

$$h\nu + H_2O + A + B \rightarrow AH_2 + BO \qquad 6.$$

AH_2 and BO merely symbolize reduced and oxidized products of photodecomposition of water attached to suitable acceptors A and B (Ruben used these symbols interchangeably with AH and BOH). AH_2, the reducing agent, would correspond either directly or indirectly to PNH_2 in reactions 3 and 5. As for BO, Ruben visualized it as an oxidizing agent, which is used in two ways. Part of it is oxidized to a peroxide and eventually yields molecular oxygen while the remainder is used in some oxidation-reduction reaction that is coupled with the esterification of inorganic phosphate into ATP.

Ruben's well-reasoned hypothesis (11) was not based on any specific evidence for the participation of phosphorus in photosynthesis but solely on consideration of thermodynamics and on biochemical concepts which gained wide currency at that time. As the subsequent discussion will show, his hypothesis in this area, has, like his pioneering work with radiocarbon, exercised a strong influence on later research in photosynthesis.

Energy-rich phosphate as the sole component of assimilatory power.—In Ruben's scheme only a portion of the light energy captured during the early photochemical reactions of photosynthesis was transformed into the pyrophosphate bond energy of ATP. The remainder was contained in the reducing agent AH_2. A more extreme proposal, however, was made shortly thereafter by Emerson, Stauffer & Umbreit (12). They suggested that the sole "function of light energy in photosynthesis is the formation of 'energy-rich' phosphate bonds." They rejected Ruben's interpretation that part of the light energy absorbed in photosynthesis is used for the reduction of carbon dioxide by a hydrogen transfer from the primary reductant, AH_2. They conceived

> the entire process of photosynthesis (the fixation, reduction and synthesis of organic molecules from carbon dioxide and, in green plants, the production of oxygen from water) as being "dark reactions" which could be accomplished without the use of light if one were able to substitute into this system the "energy-rich" phosphate compounds which actually result from the absorption of light by the chlorophyll system.

According to their views, all the light quanta absorbed during photosynthesis are first converted into the energy-rich phosphate groups of a compound exemplified by ATP. The reduction of CO_2 by the hydrogen of water to the level of carbohydrate would then proceed in a series of steps each involving an expenditure of energy not greater than that supplied by the coupled hydrolysis of one energy-rich phosphate bond (10 to 12 kcal.). On their assumption that one quantum of light gives rise to one energy-rich phos-

phate bond, a requirement of 10 to 12 quanta per molecule of CO_2 would be the minimum needed to supply the energy stored in carbohydrates (112 kcal. per C atom).

The hypothesis of Emerson *et al.* (12) was an attempt to find a common mechanism for photosynthesis and chemosynthesis. Vogler (13) had reported a little earlier that in the chemoautotrophic sulfur bacterium, *Thiobacillus thiooxidans*, it was possible to separate in time the energy-yielding oxidation of sulfur to sulfuric acid from the normally simultaneous energy-consuming assimilation of carbon dioxide into cellular substance. Sulfur was oxidized in the absence of carbon dioxide and at least a portion of the liberated energy was stored in a form which could later be used for CO_2 fixation in the absence of sulfur oxidation. The storage of the energy released by the oxidation of sulfur was accompanied by an uptake of inorganic phosphate and its conversion into organic compounds within the cell. Conversely, the later fixation of CO_2 was accompanied by a release of inorganic phosphate (14). The energy storage compound formed during the oxidation of sulfur appeared to be adenine-ribose-3-triphosphate (15). In the view of Emerson *et al.*, the essential feature common to both photosynthesis and chemosynthesis by the sulfur bacteria was the mediating role of ATP. This compound first stored in its configuration the energy derived from light or, in the case of *Thiobacillus*, from the oxidation of sulfur, and then released the stored energy to drive the endergonic reactions concerned with the assimilation of carbon dioxide.

In seeking experimental support for their hypothesis, Emerson *et al.* (12) investigated the phosphorus metabolism of *Chlorella*. The results were not very substantial on points which were especially relevant to the theory. Illuminating the cells for 90 min. either in the presence or in the absence of carbon dioxide did not significantly change the relative proportion of inorganic to organic phosphorus compounds. Light and the presence of CO_2, however, caused changes in that portion of the organic phosphate which was precipitable by barium. This fraction was further subdivided, by 7 min. hydrolysis, into a labile and a resistant portion. In the absence of carbon dioxide the relative amount of the hydrolysis-resistant portion was large in the dark but decreased upon illumination; the opposite effect was noted in the presence of carbon dioxide. These changes could not be associated with any specific phosphorus compounds, since the authors found the pattern of phosphorus fractions in *Chlorella* different from that given by the same fractionation methods with other materials such as yeast.

The phosphorylation hypothesis of photosynthesis by Emerson, Stauffer & Umbreit (12) was received with considerable criticism. In the opinion of its critics, its main shortcomings pertained to thermodynamics. Rabinowitch (16) and Gaffron (17) emphasized that the conversion of one quantum of red light containing 43 kcal. into one pyrophosphate bond of ATP of 10 to 12 kcal. represented a dissipation of energy which would increase the quantum requirement of photosynthesis to a level exceeding that experi-

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mentally observed. This objection, of course, could be met, as will be discussed later, by modifying the original hypothesis to require the formation of more than one high-energy phosphate bond per quantum of light consumed. Rabinowitch and Gaffron rejected this possibility as being incompatible with concepts of primary photochemical energy conversions.

It would seem now that the main drawback of the hypothesis of Emerson *et al.* (12) was its assumption that all, rather than only a part, of the light energy absorbed in photosynthesis is transformed into high-energy phosphate bonds, without specifying a mechanism for generating a reductant needed for CO_2 fixation. It was also known that light energy could be used for the formation of a reductant of low reducing power without the participation of phosphate, as in the Hill reaction, in which illuminated chloroplasts reduce such compounds as quinone and ferricyanide. The hypothesis of Ruben, which regarded phosphate bond energy as only one of two components of the assimilatory power generated by light, has proved itself more adaptable to later experimental advances in photosynthesis than that of Emerson *et al.* (12)

Evidence for Participation of Phosphorus in Photosynthesis; Experiments with Whole Cells

The theoretical implications of the proposals of Ruben (11) and of Emerson *et al.* (12) that phosphate bond energy is a common denominator of energy transformations in all cellular metabolism whether in the dark or in the light, stimulated a number of investigators to seek experimental verification for these at the time largely unsupported hypotheses. Most of the investigations were at first concerned with phosphorus metabolism of whole cells. The objective here was to isolate from the over-all phosphorus metabolism of the cell those phases which were directly linked with photosynthetic events. The principal experimental approaches used involved measuring the influence of light and carbon dioxide on the absorption of inorganic phosphate, its conversion to organic phosphate, and the identification, either directly or indirectly, of specific organic phosphorus compounds, especially ATP, formed at the expense of light energy.

Assimilation of inorganic phosphate.—Aronoff & Calvin (18) were the first to attack this problem with the aid of the sensitive tracer technique using P^{32}. They reasoned that if the hypotheses of Ruben and Emerson *et al.* were correct, it should be possible to demonstrate by means of radioactive phosphorus a greater rate of conversion of inorganic to organic phosphate in the light than in the dark. In experiments with tobacco leaves and *Chlorella vulgaris* cells they found with the techniques they used no evidence for this and concluded that there is "no direct connection between light and the gross formation of organic phosphorus compounds."

In contrast to this conclusion Gest & Kamen (19), also using P^{32}, found that in three organisms, *Chlorella pyrenoidosa*, *Scenedemus* (D_3 strain), and *Rhodospirillum rubrum*, there was both a considerably greater uptake and

turnover of inorganic phosphate in the light than in the dark. The authors interpreted these results as suggesting the formation of energy-rich phosphate esters during illumination but as offering no clue whether these phosphate esters were linked to photosynthetic carbon dioxide fixation.

Wassink and his collaborators (20 to 25) have concerned themselves over a period of years with the question of phosphorus metabolism in photosynthesis. In setting out to investigate this question they directed their efforts to the separation, suggested by Vogler (13), of the light-induced esterification of inorganic phosphate from the subsequent dark fixation of carbon dioxide. Their experimental technique was based on the measurement of the conversion of inorganic into organic phosphate by illuminated cells in the presence and absence of carbon dioxide (cf. 12). An increased conversion in the absence of carbon dioxide would mean that energy-rich phosphate esters were accumulated when they could not be used for the normal carbohydrate synthesis.

In their first experiments Wassink *et al.* (20) used purple sulfur bacteria (*Chromatium*). They found that in light the cells absorbed more inorganic phosphate from the external medium in the absence of carbon dioxide than in its presence. In the dark there was always a release of inorganic phosphate to the medium whether carbon dioxide was present or absent. Similar results were obtained by these authors with *Chlorella* (21). In this investigation they followed the changes in phosphorus metabolism by extracting the cells with TCA and measuring the shifts between the inorganic phosphate fraction and the organic phosphate fraction. In the presence of CO_2 the effect of light was small but in the absence of carbon dioxide illumination resulted in an appreciable conversion of inorganic into organic phosphate—much greater than in a similar experiment by Emerson *et al.* (12). In terms of total cell phosphorus the percentage conversion was 30 in the experiments of Wassink *et al.* (21) as against 5 for Emerson *et al.*

In the work cited so far the changes in inorganic phosphorus were measured after rather long exposure to light or dark. It occurred to Kandler, however, that these measurements may have masked important changes in the phosphorus metabolism occurring at the instants when light was being either turned off or on (26). He proceeded to measure, therefore, the shifts in inorganic phosphate at very short intervals following the transition from light to darkness or darkness to light. He was concerned not with increased absorption of phosphate from the external medium but rather with shifts of inorganic phosphorus (P_i) already present in the cells.

Using *Chlorella* cells suspended in a phosphate medium in the dark, Kandler found in the first 30 sec. after turning on the light, a decrease of about 20 per cent in the intracellular P_i. This decrease was followed by a rise to a stationary level, which, within approximately 2 min., stabilized itself at a value *circa* 10 per cent lower than the P_i level in the dark. These shifts, although small quantitatively, appeared to be consistent. In the light-adapted cells the change was in the opposite direction when they were

placed in the dark. Within 90 sec. of turning off the light the level of intracellular P_i increased by about 30 per cent, but soon dropped and within 3 min. reached a new equilibrium level about 10 per cent higher than that observed during illumination.

Kandler interpreted the initial decrease in intracellular P_i which accompanied the turning on of light as evidence of esterification of inorganic phosphate into energy-rich compounds by a photochemical reaction. He reasoned that the newly-synthesized energy-rich phosphate groups would be used in endergonic dark reactions (CO_2 assimilation) which would result in a release of inorganic phosphate. These dark reactions would reach their full speed within 3 to 5 minutes, after which a steady state would be established between the light-induced esterification of P_i and its subsequent hydrolysis by the dark reactions of photosynthesis. In illuminated algae at the steady state the level of intracellular P_i would thus reflect the balance between two essentially independent phosphate cycles: the esterification and hydrolysis of phosphate linked to photosynthesis and the esterification and hydrolysis of phosphate linked to respiration. When light was turned off, the level of intracellular P_i decreased because the dark reactions of photosynthesis continued for a few minutes. After this a new equilibrium between organic and inorganic phosphate was established.

Simonis & Grube (27) and Grube (28) investigated with the aid of P^{32} the effect of light and carbon dioxide on phosphorus metabolism in leaves of *Elodea densa*. The excised leaves were immersed during the experimental period in a solution containing carrier-free P^{32}. At the termination of the experiment the leaves were extracted with cold TCA. The TCA extract was further subdivided into the orthophosphate fraction (P_1) removed by precipitation with an ammonia-magnesia mixture and an organic phosphorus TCA-soluble fraction (P_2). The third fraction, included for comparison, contained the organic phosphorus compounds not extractable by cold TCA (P_3). Of greatest interest were the changes in the P_2 fraction since it included ATP and similar energy-rich organic phosphorus compounds.

Brief exposure (60 sec.) to light increased only the P_2 fraction. There was no significant change in the other two fractions. Ten min. of illumination gave a decrease of the orthophosphate fraction (P_1) and an increase in the P_2 fraction. The P_3 fraction was unchanged during this period; it showed an increase only on prolonged exposure to light (60 min.). The authors concluded that the organic phosphorus compounds in the P_3 fraction were only indirectly affected by photosynthetic events. (Compare, however, the results of Wintermans (25), discussed below, on changes of the polyphosphate fraction on long exposures to light.) As already stated, the increase in the soluble organic fraction (P_2) was accompanied by a corresponding decrease in inorganic phosphate (P_1). Both fractions were enhanced by higher light intensity. The authors concluded therefore that, under the influence of light, an esterification of inorganic phosphate into energy-rich organic phosphorus compounds had occurred.

The light-induced changes in the P_1 and P_2 fractions were greater in the presence than in the absence of carbon dioxide. However, the changes in these two fractions in the absence of an external supply of carbon dioxide were sufficiently large to suggest the possibility that photosynthetic esterification of phosphate was independent of carbon dioxide assimilation.

Relation between phosphorus and glucose assimilation.—Several investigators have sought to use the assimilation of glucose by photosynthesizing cells as a technique for the study of the effect of light on phosphorylation. Glucose and hexokinase are frequently used as a "trapping" system for newly-formed ATP, which is used to form glucose-6-phosphate in accordance with equation 7.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP} \qquad 7.$$

It would be expected that in most photosynthesizing cells glucose-6-phosphate would either be further converted to glucose-1-phosphate and starch or degraded by glycolytic enzymes. In either case glucose would have to be first phosphorylated by ATP before it could be further metabolized by either pathway. Experimental conditions could be so arranged that the supply of ATP would limit the assimilation of added glucose by green cells. If in these circumstances light could be shown to accelerate the assimilation of glucose, it might be deduced that ATP formation is directly or indirectly a consequence of photosynthesis.

Wassink *et al.* (22) compared the effect of glucose on the conversion of inorganic to organic phosphate by *Chlorella* cells in the light and in the dark. The addition of glucose had no appreciable effect in the dark, but in the light it decreased the formation of organic phosphate compounds [cf. Wintermans (25) for interpretation of methods]. This observation could perhaps be interpreted as signifying the breakdown of organic phosphate compounds accompanied by a release of inorganic phosphate, as in the conversion of glucose to starch with the aid of ATP.

The likelihood of such an interpretation is supported by the work of Kandler (29) who also used *Chlorella* cells. Kandler measured the absorption of glucose in the light and in the dark by cells which had been previously depleted of carbohydrate reserves by aeration in distilled water in the dark. Absorption of glucose from the external medium was used as a measure of its assimilation. Kandler found that when the external pH ranged from 5.0 to 7.5, *Chlorella* cells absorbed up to 25 per cent more glucose in the light than in the dark. The increased absorption of glucose was not observed in cells with intact sugar reserves or in cells which were first depleted but later supplied with glucose prior to exposure to light. The stimulatory effect of light lasted for several hours, but diminished gradually, and after 8 hr. light actually inhibited the assimilation of glucose.

Kandler's results obtained under anaerobic conditions are of special interest in connection with photosynthetic phosphorylation discussed later. In the dark, in an atmosphere of nitrogen, the cells absorbed only 5 per cent

of the amount of glucose absorbed under aerobic conditions. In the light, however, the cells absorbed under anaerobic conditions up to 85 per cent of the amount of glucose absorbed under aerobic conditions. This markedly increased anaerobic absorption in light could not be explained by an aerobic metabolism in the light made possible by the evolution of oxygen of photosynthetic origin. Parallel experiments have shown that the partial pressure of the evolved oxygen was too low to make this explanation tenable.

Kandler explained his results by postulating that the assimilation (measured by absorption) of glucose by his sugar-depleted cells was limited by the level of intracellular ATP. Exposure to light generated ATP by phosphorylation reactions directly dependent on photosynthesis. With the new supply of ATP, increased assimilation of glucose was made possible up to the point of saturating the enzymes concerned (hexokinase and phosphorylase). When the algae were high in sugar prior to the exposure to light or when they were illuminated for long periods of time, the assimilation of added glucose was no longer limited by ATP but rather by the concentration of the intracellular enzymes, which would, under these conditions, be saturated by assimilatory products. When this occurred, glucose assimilation in light could no longer be used as a measure of light-dependent phosphorylation.

Simonis & Grube (30) used a somewhat different experimental approach to demonstrate a relation between glucose and phosphate assimilation during photosynthesis. They measured the uptake of carrier-free P^{32} orthophosphate and its conversion into organic phosphorus under the influence of light by *Elodea densa* leaves. The excised leaves had been allowed just prior to the experiment to absorb glucose for short periods of time (10 min.). After a brief illumination period (3 to 10 min.) the leaves were quickly killed and extracted with TCA. The authors found that, in the light, the glucose pretreatment increased the uptake of P^{32} by the leaves into both the organic and the orthophosphate fractions. They also obtained similar results without the glucose pretreatment but by preilluminating the leaves for 10 min. and then allowing them to absorb P^{32} for a few minutes in the dark.

Simonis & Grube interpreted the increased P^{32} uptake in the organic phosphorus fraction as evidence for a light-induced phosphorylation of glucose. They suggested that the parallel increase of P^{32} in the orthophosphate fraction might have been an artifact resulting from the hydrolysis by their extraction methods of a labile organic phosphorus compound such as glucose-1-phosphate. They regarded as less likely the alternative explanation that the light-induced increase in the P^{32} of the orthophosphate fraction was an indication of an increased metabolism of glucose accompanied by the liberation of inorganic phosphate.

Identification of specific phosphorus compounds.—The investigations reported so far do not provide any direct evidence for the formation during photosynthesis of ATP or other energy-rich phosphorus compounds. The formation of such compounds during photosynthesis was inferred by the different investigators from the evidence yielded by the several indirect

experimental approaches which they use. In fact, at the time when the first investigations on the role of phosphorus in photosynthesis were launched the very occurrence of ATP in plant tissues, green or nongreen, had not yet been documented but was merely surmised from indirect evidence and by analogy with yeast and animal tissues. The search for ATP in plants was beset by experimental difficulties (5, 31). Only recently, when these were overcome, were Albaum *et al.* (31) able to demonstrate the occurrence of ATP in nongreen plant tissues, (mung bean sprouts) and in the photosynthetic alga, *Euglena gracilis* (32). More recently, Simonis & Schwinck (33) have shown by chromatographic methods the incorporation, on exposure to light, of $P^{32}O_4$ into ATP by *Ankistrodesmus* and by *Elodea* leaves.

The first direct evidence that phosphorus is implicated in some manner in the course of photosynthetic reactions was provided by the identification by Benson & Calvin (34) of phosphorylated compounds as intermediates in the assimilation of carbon dioxide. Their finding, confirmed by Gaffron *et al.* (35), that phosphoglyceric acid was the first photosynthetic product measurable by the techniques used, suggested that phosphorus compounds were involved in the earliest steps of carbon dioxide assimilation. But the identification of phosphorylated carbohydrate intermediates among the products of photosynthesis could not by itself be taken as evidence of a direct conversion, specifically linked with photosynthesis, of light into the phosphate bond energy of ATP. More direct evidence of light-induced phosphorylation was still required.

Strehler (36, 37) applied the very sensitive firefly assay method (38) to the measurement of ATP formed during photosynthesis by *Chlorella* cells. Like the experiments of Kandler (26) with inorganic phosphate, those of Strehler included measurements of changes in ATP during the induction period of photosynthesis immediately after exposing the cells to light. In Strehler's experiments the algae were previously incubated for 2 hr. in Warburg No. 9 buffer (39) under anaerobic conditions in the dark, i.e. without an opportunity for ATP formation by the respiratory chain pathway. Conditions were then changed either by admitting oxygen in the dark or by exposing the cells to light to initiate photosynthesis. The formation of ATP under the two treatments was compared. The results [Fig. 1 (36)] showed that either during anaerobic illumination or during oxygen admission in the dark, the ATP level attained a maximum after an induction period of approximately 1 min. Following the induction period, the ATP concentration in the dark under aerobic conditions stabilized itself at a maximal level; in the light the ATP level first rose and then decreased about 20 per cent in the next minute before reaching a steady state. The concentration of ATP in the steady state in the light was approximately half of that in the dark. The initial rate of ATP formation was proportional to light intensity. As for the steady state concentration at different light intensities it is difficult to reach any conclusion from the presented data [See Figures 1 and 4 (36)].

Strehler interpreted these results as providing evidence for a direct relation between illumination and ATP synthesis (36, 37). His curves of ATP levels during the induction period of illumination have sometimes been compared (1) with those of Kandler (26) on changes in inorganic phosphate in the opposite direction. However, Strehler's data are not directly comparable with those of Kandler. Strehler observed the increase in ATP on exposure to light under anaerobic conditions, and did not present data on the results of aerobic illumination, whereas Kandler found the decrease in inorganic phosphate under aerobic conditions (26). Wassink & Rombach (24) have recently corroborated Kandler's observations on the changes in concentration of inorganic phosphate during the induction period of photosynthesis under both aerobic and anerobic conditions.

Strehler's experiments do not rule out the possibility that ATP was formed in the light at the expense of oxygen of photosynthetic origin. A description of his experimental methods (36) makes no mention of precautions taken to remove, during the induction period, the newly-formed oxygen of photosynthetic origin or to measure, as in Kandler's experiment (29), the effect of the oxygen of photosynthetic origin on aerobic metabolism. His experiments therefore do not necessarily lead to the conclusion that ATP synthesis in light proceeds by a mechanism peculiar to photosynthesis.

Another argument used by Strehler in favor of a special synthesis of ATP directly induced by light was its insensitivity, as contrasted with respiratory chain phosphorylation in the dark, to low temperature. It should be noted, however, that the level of ATP formation at the low temperature (4°C.) was of the same order of magnitude whether O_2 was admitted in the dark or the cells were exposed to anaerobic illumination [Fig. 2 (36)]. At the elevated temperature (25°C.) the level of ATP formation was much higher aerobically in the dark than anaerobically in the light. It is possible that at the higher temperature respiratory chain phosphorylation occurred in the light but was limited by the low partial pressure of oxygen.

In Strehler's experiments the concentration of ATP rose during a dark period after exposure to light, regardless of whether CO_2 was present during the dark period. He inferred from this result that "the major drain on ATP in the light is not carboxylation reactions, but rather some set of processes not dependent upon the CO_2 fixation and reduction steps." His proposed role for ATP in photosynthesis will be discussed later.

Goodman, Bradley & Calvin (40) exposed *Scenedesmus* cells to carrier-free $KH_2P^{32}O_4$ for varying periods of time in the light and dark and measured the distribution of radioactive phosphorus in specific compounds by the technique of paper chromatography and radioautography. In short-term experiments (30 sec.) most of the radioactivity fixed in the dark was localized in ATP; in the light P^{32} was distributed among several compounds without any one assuming special prominence. On prolonging the exposure, ATP continued to maintain its position as the dominant product of dark exposure, whereas in the light 3-phosphoglyceric acid (PGA) became rapidly labelled.

The more rapid labeling of PGA in light than in darkness was interpreted by the authors as indicating a close relationship between incorporation of inorganic phosphate and photosynthesis. They also interpreted the lower labeling of ATP in light than in darkness as indicating that the incoporation of phosphate into photosynthetic intermediates probably proceeds via ATP. The distribution of radioactivity among intermediates other than ATP and PGA was unaffected by light and darkness.

In the investigations reported thus far, the attempts to identify the energy-rich phosphorus compounds resulting from the assimilation of orthophosphate during photosynthesis have centered around ATP. There was, however, no direct experimental evidence for regarding ATP as the compound in which radiant energy is stored after being converted into a chemical form.

When ATP in algae was measured directly in the light and in the dark by Strehler (36) and by Calvin and his co-workers (40), they found the level to be higher in the dark and interpreted this as suggesting an increased utilization of ATP during photosynthesis. The extensive work of Wintermans in Wassink's laboratory (25, 41) raises the question, however, whether in algae the conversion of light into phosphate bond energy cannot be shown more directly by following changes in phosphorus compounds other than ATP. Holzer (42) had earlier found that in *Chlorella pyrenoidosa* the bulk of hydrolyzable phosphate (7 min. 1 N HCl, 100°C.) came from metaphosphate which masked the much smaller changes in ATP resulting from exposure to light.

Wintermans (25, 41) found that illuminated *Chlorella* cells can convert orthophosphate into high molecular weight polymers of phosphoric acid, particularly in the absence of CO_2. Wintermans refers to these polymers as polyphosphates, a term which includes but is not limited to metaphosphate, i.e. the lower cyclic polymers having the composition $(HPO_3)_n$. The polyphosphates appear to be normal constituents of green cells. Thus Stich (43) reported the formation of metaphosphates by *Acetabularia mediterrenea* during normal photosynthesis, in the presence of CO_2. In Wintermans' experiments the formation of polyphosphates in light continued for several hours at a slowly decreasing rate. Polyphosphate formation in the light did not require oxygen, which afforded an important distinction from phosphate fixation in the dark which was oxygen-dependent. The polyphosphates were found to be insoluble in cold TCA but soluble in hot HCl or in dilute NaOH at room temperature.

Wintermans regards the polyphosphates as energy-rich phosphorus compounds which have been formed at the expense of light-energy, via ATP, would be the parent compound formed by primary photochemical reactions. The energy-rich phosphate groups would then be transferred from ATP and stored as polyphosphates, particularly in the absence of CO_2, when the normal photosynthetic demands on energy-rich phosphate groups have been severely curtailed. The particularly interesting feature of Wintermans' experiments, as well as of those reported earlier from the same laboratory

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(20 to 23) is that they provide the only evidence in intact cells for the storage of light energy as stable polyphosphate compounds in the absence of CO_2.

Mechanisms of Light-Induced Phosphorylations

In experiments with whole cells, photosynthesis cannot be completely isolated with certainty from concurrent metabolic processes which, like respiration, are intimately associated with phosphorus metabolism. The participation of phosphorus in photosynthesis, investigated at the cellular level, poses therefore several questions. What are the intracellular sites at which light-induced phosphorylation occurs? Have green cells evolved special mechanisms and enzyme systems which carry on phosphorylation in the light or do they use, except for the initial photochemical reactions, the same mechanisms and enzyme systems which govern phosphorylation in the dark? Is light-induced phosphorylation dependent on simultaneous assimilation of carbon dioxide?

The last question should perhaps be considered first. If it could be shown that phosphorylation in the light is merely the result of the reoxidation of products of CO_2 assimilation, it would seem probable that photosynthesis is only indirectly related to phosphorylation, through the provision of a respiratory substrate. That this is not likely to be the case has already been indicated by the evidence cited on phosphorylation by whole cells in the absence of CO_2. Let us now examine the evidence from experiments with cell-free systems pertaining to this and other aspects of the mechanism of phosphorylation in the light.

Negative evidence for light-induced phosphorylation by cell-free systems.— The first experiments on phosphorylation with cell-free systems were those of Aronoff & Calvin (18) who sought to demonstrate with P^{32} a greater rate of conversion of inorganic to organic phosphate in light than in darkness by chloroplast fragments ("grana") unaided by other enzyme systems. Evidence of phosphate esterification by illuminated chloroplast fragments would have been particularly interesting, since these preparations had no capacity for CO_2 fixation but retained the ability to utilize light energy for chemical work (the Hill reaction). However, Aronoff & Calvin obtained no conversion of inorganic to organic phosphate by chloroplast fragments.

It has already been noted that in a later investigation with P^{32} Calvin and his collaborators (40) concluded that inorganic phosphate was incorporated during photosynthesis into 3-phosphoglyceric acid, probably through reactions involving ATP. Fager (44, 45) was unable to obtain evidence in support of this conclusion in a cell-free system which retained the ability to fix CO_2 in the light into phosphoglyceric acid and which he therefore regarded as representing the enzyme components responsible for the first carbon fixation reaction in photosynthesis (34, 35). In Fager's system ATP at 10^{-3} *M* acted as an inhibitor of CO_2 fixation into PGA (44). At 10^{-4} *M* and lower concentrations, ATP caused no fixation by itself, and

did not promote fixation in the presence of several potential CO_2 acceptors, nor did it promote an exchange involving the carboxyl group of added phosphoglyceric acid (45).

Despite the negative evidence just cited, the reality of light-induced phosphorylations by cell-free systems is no longer in doubt. This conclusion is based on extensive experiments with isolated chloroplasts in which a variety of experimental conditions was tested (46, 47).

Respiratory chain phosphorylation and the Hill reaction.—In nongreen cells the principal pathway for ATP synthesis is the esterification of inorganic phosphate linked with the aerobic phase of respiration, the tricarboxylic acid cycle. This oxidative or, as it will be called here, respiratory chain phosphorylation [Holzer (42), Slater (49)] accounts for the conversion of 65 to 80 per cent of the total energy content of the substrate into the pyrophosphate bonds of ATP [Krebs (50)]. (The term respiratory chain phosphorylation is preferred here to underline the strict dependence *in vivo* of this mechanism for ATP synthesis on molecular oxygen as the terminal hydrogen acceptor.) The electron transport system in respiratory chain phosphorylation is the same regardless of the substrate that is being oxidized (50). The substrate reduces DPN (an analogous reaction with TPN has not, as yet, been experimentally demonstrated) and the reduced DPN is reoxidized by flavoproteins, cytochromes and molecular oxygen. This is represented by the following scheme (the arrows indicate the direction of the flow of electrons).

$$\text{DPNH} \rightarrow \text{flavoproteins} \rightarrow \text{cytochromes} \rightarrow O_2$$

The enzymes responsible for respiratory chain phosphorylation are contained in mitochondria. Lehninger (51) was the first to demonstrate directly, with mitochondria from animal sources, that respiratory chain phosphorylation is linked to the reoxidation of DPNH in accordance with the over-all reaction 8.

$$\text{DPNH} + H^+ + 1/2O_2 + 3\text{ADP} + 3P_i \xrightarrow{\text{mitochondria}} \text{DPN}^+ + 3\text{ATP} + H_2O \quad 8.$$

There was no direct experimental evidence that mitochondria from plant tissues were capable of carrying out reaction 8 until this gap was closed by the work in Bonner's laboratory (52). The mitochondria used by Millerd *et al.* (52) were from nonphotosynthetic tissues (mung bean seedlings) and the respiratory chain phosphorylation catalyzed by them was analogous to that mediated by mitochondria from animal sources in that no light-induced reaction was involved. Is respiratory chain phosphorylation by mitochondria also the mechanism for ATP synthesis in the light? Let us now examine the evidence which bears on this question.

Illuminated chloroplasts and chloroplast fragments were found to be capable of reducing pyridine nucleotides when coupled with appropriate enzymes (53, 54, 55). Vishniac & Ochoa (46) have showed that DPN reduced by chloroplast fragments in light can, with the aid of mitochondria from etiolated mung bean seedlings, be reoxidized by molecular oxygen with a

resultant ATP synthesis. They visualized the formation of ATP in their model chloroplast-mitochondria system as having occurred by respiratory chain phosphorylation as represented by equation 8. The role of chloroplasts was limited, in their view, to a Hill reaction, i.e., a photochemical transfer of electrons from water to DPN in accordance with equation 9.

$$H_2O + DPN^+ + \text{light} \rightarrow DPNH + H^+ + 1/2O_2 \qquad 9.$$

Once this was accomplished, ATP synthesis would proceed by the same mechanisms as were operative in nonphotosynthesizing cells. It was this concept which served as the basis of Racker's (56) model for the synthesis of carbohydrate in the dark by reduced pyridine nucleotide and ATP, in a multi-enzyme system from spinach leaves.

It is pertinent to the subsequent discussion to note that in reaction 9 one atom of oxygen is liberated whereas in reaction 8 one atom of oxygen is consumed. Theoretically, therefore, the formation of ATP by the chloroplast-mitochondria system of Vishniac & Ochoa (46) could have proceeded without an external source of oxygen. Vishniac & Ochoa observed, however, in their system a "strict dependence of phosphate exchange on the presence of oxygen." No light-induced ATP formation occurred in an atmosphere of nitrogen (46).

The generation of ATP by the chloroplast-mitochondria system (46) requires no mechanisms for phosphorylation different from those in normal respiration and peculiar to photosynthesis. DPNH and molecular oxygen are essential to both. The origin of DPNH is the only difference between the two; in one case DPNH is an intermediate product of photosynthesis, and in the other it is derived from the oxidation of a respiratory substrate. The mechanism for the reoxidation of DPNH and the resultant ATP synthesis would be the same for photosynthesis and respiration.

This postulation has certain important theoretical consequences for the over-all mechanism of photosynthesis. If it is accepted that ATP is needed for CO_2 fixation, then it follows that photosynthesis is dependent on consumption of molecular oxygen. Moreover, since, at high light intensities photosynthesis is many times more active than respiration, the consumption of molecular oxygen in the light would have to be many times greater than that in the dark. It would also be expected that green cells would contain an abundance of mitochondria, in a suitable proportion to chloroplasts, to provide the respiratory chain enzymes needed for the light-induced phosphorylation.

In an investigation of the dark metabolism of cytoplasmic particles isolated from photosynthetic tissues, Arnon & Whatley (57) concluded that the paucity of mitochondria relative to chloroplasts in green leaves made it unlikely that ATP synthesis in the light depended on respiratory chain phosphorylation. A search for the site of phosphorylation in green leaves in the light led to a re-evaluation of the role of chloroplasts in light-induced ATP formation (47, 58). The first results showed that in the light, but not in the dark, isolated whole chloroplasts, without the aid of mitochondria

or any other enzyme system extraneous to chloroplasts, vigorously synthesized ATP from inorganic phosphate and AMP or ADP when supplemented with ascorbic acid. The dispensability of mitochondria was established by separating them from the whole chloroplasts by means of differential centrifugation, washing the whole chloroplasts to remove residual mitochondrial impurities, and finding that phosphorylation by the washed chloroplasts proceeded at the same rate whether the mitochondrial fraction was added or not (47, 65).

Phosphorylation by chloroplasts differed from that by the chloroplast-mitochondria system (46) in that no requirement for added pyridine nucleotides (DPN or TPN) was observed. It seemed unlikely that this observation by the Berkeley group (47, 58) resulted from the presence of bound DPN in the chloroplast preparations which they used. In later experiments (59, 60) they have leached out, by a water treatment, most of the pyridine nucleotides from chloroplasts without affecting ATP synthesis. The addition of these coenzymes to washed (broken) chloroplasts had no effect on ATP formation but greatly stimulated CO_2 fixation in the complete system. It seems probable therefore that pyridine nucleotides are concerned with CO_2 fixation rather than with ATP formation.

In ATP synthesis by chloroplasts molecular oxygen was neither evolved nor consumed. This led to a tentative formulation (47, 58), analogous to that postulated by Ruben (11) and Kok (61), that phosphorylation by chloroplasts was linked to a recombination of an oxidized and a reduced product of the photolysis of water, as represented by equations 10 and 11.

$$\text{Light} + H_2O \rightarrow 2e^- + 2H^+ + [O] \qquad 10.$$

$$2e^- + 2H^+ + [O] + \text{ADP} + P_i \rightarrow \text{ATP} + H_2O \qquad 11.$$

$$\text{Sum: Light} + \text{ADP} + P_i \rightarrow \text{ATP} \qquad 12.$$

[O] was intended to represent an oxidized product of photodecomposition of water existing not in a free state but attached to some unknown acceptor (cf. equation 6). The brackets were also meant to signify that it was impossible to decide on the basis of the manometric evidence whether molecular oxygen was released in reaction 10 and immediately consumed in reaction 11 or whether molecular oxygen was not set free, so that ATP synthesis in reaction 11 was accomplished under anaerobic conditions. If the latter were the case it would be possible to entertain the notion that ATP formation was catalyzed by enzyme systems peculiar to chloroplasts and photosynthesis. If molecular oxygen were required for the phosphorylation it would appear probable that chloroplasts had some of the attributes of mitochondria for carrying out respiratory chain phosphorylations.

That chloroplasts were capable of catalyzing respiratory chain phosphorylation seemed unlikely. In the dark, their endogenous respiration was very low (57), and they were unable to oxidize Krebs' cycle intermediates (62). There remained, however, the discordant observation in the early phosphorylation experiments that a sustained rate of phosphorylation by

illuminated chloroplasts was obtained only under aerobic conditions (47, 58). The uncertainty as to the oxygen requirement for light-induced phosphorylation by chloroplasts was not set aside by Frenkel's finding reported shortly thereafter (63) that the particulate fraction of a sonic macerate of *Rhodospirillum rubrum* was capable of synthesizing ATP in the light under anaerobic conditions. Frenkel's system differed in several respects from the chloroplast phosphorylation system of the Berkeley group. Frenkel's particles became substrate-dependent after washing; the rate of phosphorylation was doubled upon the addition of α-ketoglutarate; phosphorylation by *Rhodospirillum* particles was insensitive to dinitrophenol (10^{-4} *M*) wheras phosphorylation by chloroplasts was inhibited by this agent (47). Certain similarities between the two systems were noted: phosphorylation in both cases showed little response to the addition of pyridine nucleotides and was inhibited by *o*-phenanthroline (see later discussion). What seemed at the time to weigh most, however, against an equivalence of the two systems was the fact that normal photosynthesis in *Rhodospirillum* cells proceeds anaerobically without any evolution of oxygen. Photosynthetic metabolism in these bacteria completely suppresses aerobic respiration (cf. 64). The light-induced phosphorylation in *Rhodospirillum* would therefore be expected to be anaerobic, but it could offer no proof for anaerobic phosphorylation by chloroplasts derived from such an eminently aerobic plant as spinach.

Further investigation of the light-induced phosphorylation by spinach chloroplasts resulted in the identification of the cofactors involved and soon yielded evidence of its anaerobic character (65, 66, 67). When these were added and conditions were so arranged that traces of oxygen originally present or possibly formed during the reaction were eliminated (shaking in a nitrogen atmosphere in the presence of chromous chloride), phosphorylation proceeded at rates in no case lower and in most cases substantially higher than those observed aerobically. It was concluded, therefore, that ATP synthesis by chloroplasts represents an anaerobic synthesis of pyrophosphate bonds at the expense of light energy by an enzyme system peculiar to photosynthesis (66, 67, 68). The light-dependent ATP synthesis by chloroplasts was named photosynthetic phosphorylation to distinguish it from the respiratory chain phosphorylation in the dark. It then seemed probable that, despite some of the differences already noted, photosynthetic phosphorylation by chloroplasts and by *Rhodospirillum* particles proceeds by analogous mechanisms. Some of the known properties of photosynthetic and respiratory chain phosphorylation are summarized in Table I.

The cofactors of photosynthetic phosphorylation identified so far are Mg^{++}, FMN, vitamin K, and ascorbate. All of these are known components of green leaves, and some, like vitamin *K*, are characteristically concentrated in the chloroplasts (69). Mg^{++} probably has a catalytic function in the transfer of phosphate groups (70). The other cofactors may serve as electron carriers. The quantities in which they are required indicate that these substances act as catalysts and not as substrates (66, 67). The identity of the electron carriers above ascorbate is unknown. They may prove to be

TABLE I

COMPARISON OF RESPIRATORY CHAIN PHOSPHORYLATION WITH PHOTOSYNTHETIC PHOSPHORYLATION (62).

	Respiratory chain phosphorylation	Photosynthetic phosphorylation
Site in the cell	mitochondria	chloroplasts
Initial electron acceptor	DPN	FMN*
Terminal electron acceptor	O_2	[O]†
Oxidation of Krebs cycle intermediates	Yes	No
Sensitivity to Antimycin A	Yes	No
Sensitivity to Gramicidin	Yes	No
Sensitivity to methylene blue	Yes	Yes
Sensitivity to dinitrophenol	Yes	Yes
Ascorbate requirement	?	Yes
Vitamin K requirement	Yes‡	Yes

* Or vitamin K.

† Oxidized product of photodecomposition of water, not molecular oxygen.

‡ Based on the work of Martius (82).

components of a cytochrome system (71, 72, 73), although other possibilities cannot be *a priori* excluded.

In earlier experiments, the Berkeley group (47, 58, 62, 65) obtained active photosynthetic phosphorylation only with whole chloroplasts. In later experiments (59, 60) they found that on destroying the structure of the whole chloroplasts by treatment with water their capacity for photosynthetic phosphorylation was unimpaired when the known cofactors were added (59). The response of broken chloroplasts to the addition of these cofactors was similar to that of whole chloroplasts (59, 60).

Photosynthetic phosphorylation by broken chloroplasts depended on the addition of cofactors only. The enzymes involved in photosynthetic phosphorylation were apparently unaffected by the disruption of chloroplast structure by the water treatment and remained bound to the broken chloroplast particles.

Since the energy content of one quantum of red light is about 43 kcal. (per Einstein) and that of the pyrophosphate bond of ATP 12 kcal. (50), it is evident that photosynthetic phosphorylation, to be efficient, must convert the energy of one quantum of light into the energy of more than one pyrophosphate bond of ATP. As was discussed earlier, Rabinowitch (16) and Gaffron (17) considered this unlikely and used this argument for rejecting the theory of Emerson *et al.* (12). Nevertheless, it seems that the conversion of the energy of one light quantum into more than one pyrophosphate bond would be theoretically possible if the recombination of the products of water photolysis [H] and [O], occurred not in one but in several successive steps, each step transforming a portion of the electron energy into phosphate bond

energy, in a manner analogous to that envisaged by Lipmann (74) for the reoxidation of DPNH by molecular oxygen during respiratory chain phosphorylation. It is conceivable however, that photosynthetic phosphorylation may be thermodynamically inefficient but physiologically important when light is abundant as during periods of strong illumination.

A scheme for an "electron ladder" mechanism for photosynthetic phosphorylation has been proposed (59, 60, 62, 67, 68). The role of the primary electron acceptor has been tentatively assigned to FMN. It is possible that *in vivo* vitamin K rather than FMN serves in that capacity. Wessels has recently postulated, on theoretical grounds, such a role for vitamin K in photosynthesis (75). With either FMN or vitamin K as the primary electron acceptor, the photolysis of water could be accomplished by one quantum of red light.

Computations based on known redox potentials (76) support the conclusion reached from biochemical evidence that in photosynthetic phosphorylation either FMN or vitamin K rather than DPN is the primary electron acceptor. Assuming a one quantum process and an oxidation level of [O] equal to that of molecular oxygen at 0.2 atm., enough energy would be available in one quantum of red light to maintain the ratio of FMN_{red}/FMN_{ox} at approximately five at pH 7; the comparable ratio for vitamin $K_{1\ red}/K_{1\ ox}$ would be approximately 1000. By contrast the comparable ratio for DPN_{red}/DPN_{ox} would be approximately 0.00001.

The assumption that the oxidation level of [O] is at the level of molecular oxygen is an arbitrary one. A lower oxidation level for [O] would allow, for the expenditure of the 43 kcal. contained in one quantum of red light, an acceptor more reduced than FMN for the hydrogen resulting from the photolysis of water. Thermodynamically, it is, of course, only the difference between the potentials of the reduced and oxidized products of the photolysis of water that must not exceed the energy equivalent of 43 kcals. for a two electron transfer (*circa* 0.9 volts).

Independence of photosynthetic phosphorylation from CO_2 fixation.—Whole chloroplasts were also found to fix CO_2 in light (47). Among the products of CO_2 fixation were identified starch and soluble phosphorylated sugars [Allen *et al.* (77)]. It became important, therefore, to determine whether the light-induced ATP synthesis proceeded at the expense of energy released by the reoxidation of partly or wholly reduced products of photosynthetic CO_2 fixation [Franck (78)]. ATP formation was found to be independent of CO_2 fixation and to proceed unimpaired when CO_2 was excluded from the reaction vessels (47, 58, 65).

The independence of photosynthetic phosphorylation from CO_2 fixation was also demonstrated by experiments with broken chloroplasts and with inhibitors. Chloroplasts broken by water treatment were able, on the addition of cofactors, but without the addition of any other enzymes, to synthesize ATP in the light. The same chloroplast preparations, however, lost completely their capacity for CO_2 fixation. To restore it, it was necessary to add the enzymes leached out by the water treatment. The results left no

doubt that in broken chloroplasts photosynthetic phosphorylation proceeded without the enzymes needed for CO_2 fixation (59, 60).

All inhibitors of photosynthetic phosphorylation were also inhibitors of CO_2 fixation by chloroplasts. Among these were *o*-phenanthroline, *p*-chloromercuribenzoate, dinitrophenol, methylene blue, and cyanide (47, 62). The inhibition by *p*-chloromercuribenzoate was reversible by glutathione, suggesting the participation of sulfhydryl groups in both photosynthetic phosphorylation and CO_2 fixation. Among the sulfhydryl inhibitors tested, however, iodoacetamide and sodium arsenite were sharply inhibitory of CO_2 fixation at concentrations which were without effect on photosynthetic phosphorylation (47, 62). It is interesting to note that antimycin A and gramicidin, which are known to inhibit respiratory chain phosphorylation by mitochondria, were without effect on photosynthetic phosphorylation by chloroplasts (62).

The observed independence of photosynthetic phosphorylation by isolated chloroplasts from CO_2 fixation is in good agreement with similar observations, discussed earlier, on light-induced phosphorylations by intact cells, in the absence of carbon dioxide.

Can chloroplasts catalyze respiratory chain phosphorylation?—Effective as photosynthetic phosphorylation may be as a mechanism for generating ATP in the light, it obviously cannot function in the dark. In aerobic plants a respiratory chain phosphorylation mechanism must take over. Zöllner (79) has already shown respiratory chain phosphorylation in the dark in intact *Chlorella* cells. The question arises whether in green cells photosynthetic phosphorylation, which generates ATP independently of molecular oxygen in the light, and respiratory chain phosphorylation, which generates ATP aerobically independently of light, occur at the same or at different sites. Are chloroplasts the site of all phosphorylations in green cells, in the light as well as in the dark, or is their phosphorylating function limited only to photosynthesis and that of mitochondria to respiration?

Several observations suggest that chloroplasts are adapted solely to the photosynthetic phosphorylation. Phosphorylation by chloroplasts was found to be strictly light-dependent (47, 58, 59, 65, 68). In the dark, chloroplasts exhibited practically no endogenous oxygen uptake (57), nor was any oxygen absorption observed when they were incubated with 10 micromoles of each of the following substrates: acetate, pyruvate, malate, succinate, citrate, ascorbate, and malate plus pyruvate. The addition of ATP and of pyridine nucleotides was also without effect (62).

The observations cited above were made with whole chloroplasts. Recently however, Ohmura (80) presented evidence for respiratory chain phosphorylation by chloroplast fragments in the dark. In the light Ohmura's particles showed no capacity for photosynthetic phosphorylation. When his green particulate fraction from spinach leaves was prepared in a citrate buffer, it consumed oxygen in the dark in the presence of organic acids, of which citrate was the most effective. Simultaneously the inorganic phosphate in the external medium decreased.

Ohmura (80) interpreted his results as suggesting that the photosynthetic phosphorylation reported by the Berkeley group (47) was accomplished by a respiratory chain mechanism present in their whole chloroplast preparations and also retained in his own chloroplast fragments. However, an alternative explanation of Ohmura's results seems also possible, namely, that his chloroplast fragments were mixed with mitochondria and that these latter particles accounted for the respiratory chain phosphorylation which he observed. This alternative interpretation is based on consideration of methodological factors. Whole chloroplasts are larger than mitochondria and can therefore be sedimented at low centrifugal forces, free from major contamination by mitochondria. When macerating leaves some chloroplasts, the proportion varying with the technique used, get broken. These chloroplast fragments become mixed with other cytoplasmic particles of similar size. Thus the application of higher centrifugal force to a leaf macerate from which whole chloroplasts have been removed yields a green fraction, which although usually called "chloroplast fragments" can be shown by several independent lines of evidence to consist of a mixture of chloroplast fragments with small nonchloroplast particles corresponding to mitochondria [McClendon (81); Arnon & Whatley (57)].

This whole question was reexamined (59, 62, 68) by preparing from spinach leaves whole chloroplasts and "chloroplast fragments" similar to Ohmura's preparation. Whole chloroplasts prepared in a citrate buffer showed vigorous photosynthetic phosphorylation under anaerobic conditions but only negligible respiratory chain phosphorylation. By contrast, the "chloroplast fragments" had, in agreement with Ohmura's results (80), an appreciable capacity for respiratory chain phosphorylation and a reduced one for photosynthetic phosphorylation. When whole chloroplasts were combined with the "chloroplast fragments" the activity of this mixed preparation ("all particles") for the two types of phosphorylation was intermediate between whole chloroplasts and chloroplast fragments [Table IV, (62)].

It seems reasonable to conclude, therefore, that in green cells, the generation of ATP occurs at two distinct sites and is catalyzed by two enzyme systems: photosynthetic phosphorylation in chloroplasts and respiratory chain phosphorylation in the smaller cytoplasmic particles, presumably mitochondria (59, 62, 68). The capacity of any particulate fraction from green leaves for the two types of phosphorylation would thus depend on the proportion of the active particles of each type present.

This conclusion does not rule out the possibility that certain individual steps in the electron transfer during photosynthetic phosphorylation are similar to those in respiratory chain phosphorylation. In both systems phosphorylation is coupled with electron transfer reactions. The catalysts so far identified for photosynthetic phosphorylation, Mg^{++}, FMN, vitamin K, and ascorbate, are either known to be or are suspected of being involved in respiratory chain phosphorylations. Of special interest in this connection are the recent findings of Martius (82) on the participation of vitamin K in respiratory chain phosphorylation by mitochondria.

Fresh support for the idea of duality of phosphorylating sites in green cells is found in a recent note by Ohmura (48), although here again the interpretation of the data by the reviewer differs from that of the author. In these experiments leaf particles were prepared in a buffer containing ascorbate instead of citrate and were separated into two fractions: whole chloroplasts and chloroplast fragments. Whole chloroplasts had the capacity for photosynthetic phosphorylation and none for respiratory chain phosphorylation in the dark with citrate as substrate, whereas chloroplast fragments were capable of carrying out both types of phosphorylation. Ohmura suggested the impermeability of substrate (citrate) to the site of phosphorylation in whole chloroplasts as the reason for their failure to phosphorylate in the dark in the presence of oxygen. However, as already noted, such relatively large molecules as adenylates, FMN, and vitamin K increase photosynthetic phosphorylation by whole chloroplasts. Ohmura's results could be alternatively explained by the presence in the "chloroplast fragments" fraction, but not in the whole chloroplasts, of an admixture of mitochondria. In the mixed preparation [compare table IV, (62)] the chloroplast component would catalyze the photosynthetic phosphorylation, and the mitochondrial component the respiratory chain phosphorylation.

Another point of similarity between the results of Ohmura (48) and those of the Berkeley group (47, 65, 66, 67, 68) are his findings that ascorbate and FMN are needed for photosynthetic phosphorylation, and that the addition of DPN and TPN was without effect. An apparent difference between the results from the two laboratories was that in Ohmura's experiments higher rates of photosynthetic phosphorylation were not obtained under anaerobic conditions. This difference does not seem to be substantial. Appreciable rates for photosynthetic phosphorylation under aerobic conditions were also observed by the Berkeley group (65), and certain of their recent observations suggest that the increase observed by the elimination of oxygen depends on experimental conditions. The important point, which does not seem to be contradicted by Ohmura's evidence (48), is that photosynthetic, unlike respiratory chain, phosphorylation is independent of molecular oxygen and can proceed unhampered under anerobic conditions.

Measurements of light-induced phosphorylations.—In the experiments with cell-free systems the progress of light-induced phosphorylation was measured either by the disappearance of phosphate from the reaction mixture or the isolation and identification of the ATP formed from inorganic phosphate. Vishniac & Ochoa (46) determined the exchange of $P^{32}O_4$ with the two terminal groups of added ATP; the radioactivity in the ATP was then transferred to glucose-6-phosphate by incubating ATP^{32} with glucose in the presence of hexokinase and myokinase. Frenkel (63) measured the disappearance of inorganic phosphate from the reaction mixture either in the presence of trace amounts of adenosine polyphosphates and a hexokinase system (mannose rather than glucose was the phosphate acceptor) or in the presence of substrate amounts of ADP. In the latter case the disappearance of orthophosphate from the reaction mixture could be stoichometrically

accounted for by the increase in the acid hydrolyzable phosphate of the esterified product.

The Berkeley group (47, 65) used $P^{32}O_4$ and substrate amounts of AMP and identified ATP as the product of photosynthetic phosphorylation directly by adsorption on Norite, followed by hydrolysis of labile phosphate or by using the isolated radioactive ATP to convert glucose to radioactive glucose-6-phosphate by the hexokinase reaction. In other experiments they used catalytic amounts of AMP with glucose and hexokinase as an ATP trapping system during the progress of photosynthetic phosphorylation. In both cases the product of the hexokinase reaction, the radioactive glucose-6-phosphate, was identified chromatographically. Ohmura (48, 80) measured the phosphorylation reactions by the disappearance of inorganic phosphate from the reaction mixture, and in the case of the respiratory chain phosphorylations by the uptake of oxygen.

Takashima & Mitsui (83) found that a spinach leaf homogenate exposed to light reduced the liberation of inorganic phosphate from α- or β-glycerophosphate. The effect of light was ascribed to a transphosphorylation catalyzed by illuminated chloroplast fragments ("grana"), between glycerophosphate and some isolable, but as yet undefined, phosphate acceptor. A number of substances, including sugars and AMP, were ineffective as phosphate acceptors in this reaction.

Mechanism of Light-Induced Phosphorylations in Whole Cells

In most schemes of photosynthesis *in vivo* which included the participation of ATP, the mechanism for its generation was envisaged as involving respiratory chain phosphorylation with molecular oxygen [see, for example, Figure 7 (84)]. This view was supported by the previously discussed experiments with chloroplast-mitochondria models (46). Investigations of photosynthesis at the cellular level, have, until recently, provided little direct evidence for a mechanism of light-induced phosphorylations.

Well known inhibitors of respiratory chain phosphorylation also inhibit photosynthesis in intact cells. Of special interest in this connection is dinitrophenol, widely used as an uncoupling agent for respiratory chain phosphorylation, where it suppresses ATP formation without affecting respiration. Holzer (42, 85) found that in *Chlorella* cells dinitrophenol at low concentrations sharply inhibited photosynthesis without affecting respiration. He interpreted these results as indicating the dependence of CO_2 fixation on ATP, the ATP being generated in the uninhibited cells by a respiratory chain phosphorylation mechanism operating in the light. In the presence of dinitrophenol, phosphorylation in the light as well as in the dark, was uncoupled from the electron transfer reactions between the reductant and molecular oxygen. Newburgh & Burris (86) observed in the presence of dinitrophenol, a reduction in the total fixation of $C^{14}O_2$ by tobacco leaves. Of the total amount fixed in the presence of dinitrophenol, a greater proportion of $C^{14}O_2$ was incorporated in the nonphosphorylated compounds, particularly alanine, than in the phosphorylated ones, such as phosphoglyceric

acid and hexose diphosphate. The authors concluded that dinitrophenol interfered with the respiratory chain in the light and thus reduced the synthesis of ATP. Wintermans (25) found that in the *Chlorella* cell dinitrophenol inhibited the formation of polyphosphates in the light in the absence of CO_2 to the same degree as the over-all rate of photosynthesis. This he interpreted as suggesting an interference by dinitrophenol with a phosphorylation step common to both processes. The high-energy phosphate compound formed, presumably ATP, could, in his view, either be converted to a polyphosphate and stored in the absence of CO_2 or used in photosynthesis for CO_2 assimilation.

Granted the uncoupling effect of dinitrophenol on phosphorylations both in the light and in the dark, it does not necessarily follow that all the enzymatic mechanisms in the two processes are the same. It is conceivable that the effect of dinitrophenol is the result of blocking an identical or similar phosphorylation reaction which is coupled however, to two different electron transport systems, one peculiar to photosynthesis and characterized by independence from molecular oxygen and the other characteristic of respiration and dependent on molecular oxygen as the terminal electron acceptor. This conclusion was drawn from the experiments, discussed earlier, on photosynthetic phosphorylation by isolated chloroplasts. It is interesting to record therefore a similar conclusion reached by Kandler on the basis of experiments with intact *Chlorella* cells (87).

In his earlier experiments (29), Kandler had correlated phosphorylation with the assimilation of glucose by depleted *Chlorella* cells in the light. In his most recent investigation he set out to compare the effects of a series of inhibitors on the assimilation of glucose in the light and in the dark. Arsenate inhibited to the same degree in the light and in the dark, suggesting a general competitive inhibition of phosphate transfer reactions. Other inhibitors were divided into two groups each affecting one of the two phosphorylation mechanisms known to operate in cellular metabolism in the dark. Group 1, consisting of iodoacetate, fluoride, fluoroacetate, arsenite, and malonate, was selected for its effect on substrate phosphorylation and Group 2 including dinitrophenol, azide, and cyanide, for its inhibitory effect on respiratory chain phosphorylation. Kandler reasoned that an equal degree of suppression of glucose assimilation in the light and in the dark by the chosen inhibitors would indicate that the same mechanism of phosphorylation was involved. If, however, both sets of inhibitors reduced glucose assimilation in the light less than in the dark, the conclusion would be drawn that the generation of high-energy phosphate groups in light proceeds by a special mechanism different from either substrate or respiratory chain phosphorylation, the two systems known to operate in the dark.

Kandler found that in the light the inhibitors in both groups reduced glucose assimilation less than in the dark. He concluded therefore that in the light, high-energy phosphate groups were formed, in the main, not by the esterification of substrates, such as products of photosynthetic CO_2 fixation, (sensitive to inhibitors in Group 1), nor by respiratory chain phos-

phorylation (sensitive to inhibitors in Group 2), but by a special mechanism peculiar to photosynthesis. The photosynthetic phosphorylation would be closely linked to the early photochemical reactions and independent of CO_2 fixation. He found support for this conclusion in the inhibition of phosphorylation by *o*-phenanthroline, a well-known inhibitor of the Hill reaction and, in his latest investigation (88), in the insensitivity of phosphorylation in the light to short-time exposures (2 min.) to 0.1 *M* cyanide. [These brief exposures to high concentrations of cyanide are not comparable to the long-term (over 1 hr.) cyanide treatment given in the experiments of Arnon *et al.* (62)].

The conclusion which Kandler reached from experiments with whole *Chlorella* cells about the existence of a special mechanism for photosynthetic phosphorylation, independent of respiratory chain phosphorylation, is thus in close agreement with that reached by the Berkeley group (62, 66, 68) from experiments with isolated chloroplasts. The apparent discrepancy (suspected by Kandler to have been the result of different experimental conditions), that glucose assimilation in light was independent of molecular oxygen (29) whereas photosynthetic phosphorylation was not (47), was set aside by the evidence from later experiments with chloroplasts (65, 66, 68). Further agreement is found in Wintermans' result that light-induced phosphorylation in *Chlorella* cells was favored by anaerobiosis in the absence of CO_2, i.e. under conditions when oxygen of photosynthetic origin could not have been of much importance. In the dark, phosphate fixation required oxygen. The earlier observations of Strehler (36) on ATP formation by *Chlorella* cells illuminated anaerobically have already been discussed.

There is thus evidence from experiments with whole cells as well as with isolated chloroplasts that photosynthetic phosphorylation is independent of molecular oxygen and respiration. The elucidation of the detailed mechanism of phosphorylation in the light must, of course, await further study. Among the proposals made so far are those of Kandler (87) which place the phosphorylation reaction before the "splitting of water" step. The Berkeley group (47, 59, 60, 62, 66, 68) retains, as the simplest explanation of the facts observed so far, the idea that photolysis of water is the primary photochemical reaction and that phosphorylation is linked to the recombination of the reduced and oxidized products of photodecomposition of water. Kandler (87) rejected the recombination theory as implying a reoxidation of the photoreductant by respiratory chain phosphorylation with molecular oxygen, which he rightly considers as improbable. But the recombination theory, as modified by the Berkeley group, excludes respiratory chain phosphorylation and molecular oxygen and is not in conflict with the evidence obtained from experiments with whole *Chlorella* cells. The salient point on which there seems to be agreement is that photosynthetic phosphorylation is catalyzed by a special enzyme system peculiar to green cells and distinct from systems responsible for respiratory chain phosphorylation. As was already emphasized earlier, this conclusion does not rule out the possible identity of some individual steps in the two processes.

Role of Light-Induced Phosphorylations in Photosynthesis

As was already discussed, Ruben (11) assigned to phosphate bond energy a role in two reactions: (*a*) the initial fixation of CO_2 as the carboxyl group of an organic acceptor, and (*b*) the phosphorylation of the carboxyl group to make its reduction to an aldehyde thermodynamically possible by a reductant, AH_2, with a reducing potential not greater than that of pyridine nucleotide. The pyridine nucleotide reductant was, in his view, a product of the primary photolysis reaction in photosynthesis in which phosphorylation reactions played no part (see reaction 6).

In his first paper Kandler (26) considered the question how the energy contained in one quantum of red light (43 kcal. per Einstein) could be used for the generation of ATP coupled with a photolysis of water. He proposed two schemes. In his scheme I, one electron (hydrogen) was raised to a very high reducing potential and then partly reoxidized to an intermediary level, corresponding to pyridine nucleotide. This partial reoxidation was coupled with the generation of energy-rich phosphate. The nature of the initial electron acceptor was not surmised. In scheme II, Kandler apportioned the energy of one light quantum to the transfer of two electrons. He considered first the possibility that pyridine nucleotides could be the initial electron acceptors. However, to accomplish the reduction of pyridine nucleotides to the 50 per cent level ($PN_{red}/PN_{ox}=1$) an expenditure of energy equal to about 50 kcal. is required. Kandler reasoned therefore [scheme II (26)] that, on theoretical grounds, the primary hydrogen acceptor in a two electron transfer reaction was more likely to be a flavin than a pyridine nucleotide. The reduction of a flavin to the 50 per cent level would require only 40 kcal. He suggested that a portion of the reduced flavin ($FMNH_2$) was oxidized with a concomitant formation of ATP by respiratory chain phosphorylation. A portion of the ATP thus formed would then be used to generate the stronger reductant (DPNH) by reducing DPN with $FMNH_2$. This latter reaction, which is a reverse of oxidative phosphorylation, could proceed only at the expense of the energy liberated by the hydrolysis of ATP. The DPNH formed in this way would then, jointly with ATP, bring about the assimilation of CO_2, as was originally proposed by Ruben (11).

Kandler has thus accepted Ruben's basic hypothesis, also elaborated by Kok (61), that CO_2 assimilation in photosynthesis involves the joint participation of ATP and a reductant at the pyridine nucleotide level. Kandler added the idea that ATP, formed by a light reaction, could be used, by a reversal of oxidative phosphorylation, to generate a strong reductant. Kandler's scheme II was also invoked, with some modification, by others (75, 89), but Strehler (36) extended it to a more extreme proposal. He suggested that ATP takes no direct part in CO_2 reduction but that its sole function in photosynthesis is the production of a strong reductant. According to his formulation,

> the photochemical process produces a reductant of intermediate reducing potential: (*a*) part of this primary reductant is consumed in a coupled oxidative phosphorylation

and (*b*) the phosphoric anhydride bond energy conserved in process (*a*) is used to raise the potential of the remaining reductant in a stepwise manner to the potential of the carbohydrate system (36).

To accomplish the reduction of a carboxyl group, as in phosphoglycerate, to an aldehyde without the participation of ATP, would, as already mentioned, require a stronger reductant than DPNH. No convincing evidence for the existence of such a reductant in green cells as postulated by Strehler has yet come to light. On the other hand, direct evidence for the probable participation of pyridine nucleotides in photosynthesis has recently come from the measurements by Duysens (90) of spectral differences on illumination of *Chlorella* and *Porphyridium* cells and from experiments on photosynthetic CO_2 assimilation by broken chloroplasts (59, 60). In the latter experiments, evidence was also obtained for the participation of ATP in CO_2 assimilation by broken chloroplasts.

The hypotheses of Ruben (11), Kok (61), and Kandler (26) are also incorporated in the proposed role of photosynthetic phosphorylation in photosynthesis by isolated chloroplasts (59, 60, 68) from green plants. Since complete photosynthesis was attained with isolated chloroplasts, the Berkeley workers regard these cytoplasmic structures as containing all the enzymes required for this process. They consider these enzymes to be divided into three main groups, each controlling an increasingly complex phase of photosynthesis: photolysis of water, photosynthetic phosphorylation, and CO_2 fixation. Whole chloroplasts contain all three groups of enzymes (59, 62, 68). Broken chloroplasts (59, 60) contain only two, since the group of CO_2-fixing enzymes is leached out by treatment with water. The phosphorylating enzymes were found to be insoluble and remained bound in the particles with the enzymes of photolysis.

In vivo, according to the Berkeley workers, photolysis is linked either with phosphorylation resulting in the production of ATP and the reconstitution of water, or with CO_2 fixation, resulting in the evolution of oxygen, and the reduction of CO_2 to the level of carbohydrate. CO_2 fixation requires the participation of all three groups of enzymes, phosphorylation requires two, whereas photolysis of water can proceed, in nonphysiological situations, without the others provided an artificial hydrogen acceptor is supplied (Hill reaction). They accepted the earlier suggestions that ATP generated by photosynthetic phosphorylation is required to aid in the thermodynamically difficult steps of CO_2 fixation and the generation of reduced pyridine nucleotides since the addition of ATP and TPN or DPN markedly increased CO_2 assimilation by reconstituted broken chloroplasts (59, 60).

The first experiments on photosynthetic phosphorylation (47) already suggested that in isolated chloroplasts phosphorylation might compete with CO_2 fixation for the reduced product of water photolysis. Further support for this idea was found in the consistent inhibition of CO_2 fixation not only by inorganic phosphate (47) but also by one of the cofactors of photosynthetic phosphorylation, vitamin K. It is conceivable that in isolated chloro-

plasts there is a lack of the normal regulatory mechanisms (one of these may be the level of inorganic phosphate) which control the orderly distribution of the primary photoreductant to the phosphorylating and the CO_2-fixation pathways.

The proposed increasing order of complexity for the three chloroplast reactions was supported by their separation by means of differential inhibitors (47, 62, 68). It was possible to inhibit a more complex phase of photosynthesis without affecting the simpler one which preceded it and, conversely, inhibition of a simpler phase of photosynthesis was invariably paralleled by an inhibition of the more complex phase which followed it. Thus CO_2 assimilation was abolished without impairing photosynthetic phosphorylation and photolysis. The enzymes catalyzing photosynthetic phosphorylation were inhibited without affecting photolysis.

Extracellular photosynthesis by chloroplasts proceeded in the absence of respiration, oxidative phosphorylation, or other cellular processes requiring the consumption of molecular oxygen. This adds credence to the view that light-induced phosphorylations are catalyzed by a complex of enzymes, especially adapted to photosynthesis and localized, not in the mitochondria, but in the chloroplasts, in close proximity to chlorophyll. The photosynthetic phosphorylating system of green cells retains a high activity outside the living cell, does not depend on the maintenance of chloroplast structure and seems to be rather free of ATP-hydrolyzing enzymes. These facts augur well for continued progress which may lead to a better understanding of the mechanism not only of photosynthetic phosphorylation but also perhaps of all other phosphorylations in living cells.

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ABSORPTION AND TRANSLOCATION OF PLANT REGULATORS[1,2]

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Naturally-occurring auxins, of which indoleacetic acid is the best known example, have a striking apex-to-base polar transport. This may have led to the belief that all plant regulators have a rather specific mode of absorption and of transport. However, since plant regulators now (5) include a wide variety of organic compounds this seems most unlikely.

Many of the phenomena which are involved in the absorption and translocation of regulators in plants are well known. However, a unified picture seems lacking. It is a rather new field, which one will not find discussed in standard texts of plant physiology. The significance of this subject is more than academic. In recent years sprays have been applied to plants for a multitude of practical purposes (106a).

New techniques have helped in the understanding of the principles of absorption and translocation. Enzymatic digestion is used for the isolation of the plant cuticle, a principal barrier through which materials must pass. Electron microscopy is showing its detailed structure. Radioactive tracers and chromatography have been useful in the microanalysis of materials, revealing their distribution on and in the plant. The author has attempted to outline the broad principles involved.

Physical Aspects of Permeation

Structure of the cuticle.—Plant parts exposed to air are covered by a fatty layer, the cuticle. In lower forms such as the mold *Phycomyces*, the cuticle is a simple, thin membrane of low elasticity and of poor adherence to the cell wall (87). In higher plants the cuticle is usually complex, as sketched in Figure 1. It has a framework of cutin with flat wax platelets embedded within it. This structure has been likened to a rubber sponge in which the holes are filled with wax (86, 93).

On the outside there is extruded a large number of wax rodlets, which, under the electron microscope (71, 93), give the outside of a leaf the appearance of a stubble beard. The wax rodlets have the general dimensions of mitochondria, i.e., of the order of 1 to 10μ in length. They are much longer in sugar cane. On the inside, where the cuticle merges into the cellulose of the

[1] The literature survey for this review was concluded in December, 1955.

[2] The following abbreviations and common names have been used: IA, 3-indoleacetic acid; NA, 1-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MCPA, 2-methyl-4-chlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; TIBA, 2,3,5-triiodobenzoic acid; Amine 220, 1-hydroxyethyl-2-heptadecenyl glyoxalidine; Schradan, octamethylpyrophosphoramide. For definitions of regulators, auxins, hormones, etc. please refer to (5).

epidermal cell walls, pectin in often present in the form of a thin layer (81). This layer is used by invading parasitic fungi, which digest it with their pectic enzymes. Preparations of similar enzymes serve to isolate films of cuticle from leaves for study in the laboratory (74).

Although the cuticle may vary greatly in thickness and in degree of wax development, it covers all parts of the shoot, including the apical meristem.

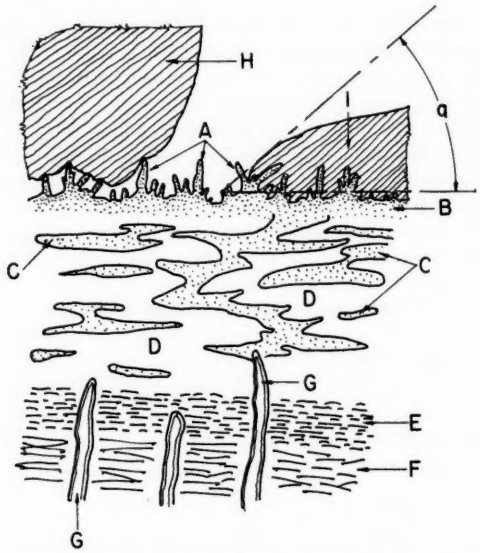

FIG. 1. Diagrammatic presentation of a cross section of a leaf cuticle. A: Tiny wax rodlets—these are probably not branched. B: External cutin layers impregnated with wax molecules. C: Wax platelets, interconnecting. D: Cutin, the semi-polar frame of the cuticle. E: Pectin layer. F: Cellulose of the outer epidermal wall. G: Plasmodesmata surrounded by plasma membrane. H: Water droplet without detergent; air film below it prevents contact with cuticle proper. I: Water droplet containing wetting agent. Note that the contact angle (a) is much smaller than in the drop H. [After Doctor's Thesis by W. H. Orgell (75).]

Cutin (33) and also waxy material (92) have been identified on the free surfaces of the leaf mesophyll cells and on the inner walls of the epidermis where these are exposed to internal air spaces. The internal cuticle is continuous with the external cuticle through the stomatal apertures, whose bounding cells, the guard cells, are covered with a cuticle on their free surface.

Clearly, cuticle is the first barrier which spray-chemicals must pass. Even when sprays penetrate through the stomata, their active ingredient will still have to pass the cutin layer which lines the interior air spaces of the leaf.

XUM

Chemical composition of the cuticle.—The wax rods of the cuticle of sugar cane stems contain molecules of higher alcohols possessing 28 carbon atoms (C_{28}). These molecules lie perpendicular to the longitudinal axis of the rods (56). The rods are pushed up from below and crystallization of the wax probably takes place at the surface of the cuticle. The cotton cuticle contains C_{28} and C_{30} alcohols (81). Rose-petal wax has also primary alcohols, and the corresponding saturated ketonic and hydroxy acids. These are mostly in the C_{24} to C_{30} range. It is interesting to note that all of these components have an even number of carbon atoms (21). However the paraffins, ketones, and secondary alcohols of wax, which are probably formed from the acids by decarboxylation, have an odd number of carbon atoms.

Wax is synthesized continuously during the life of the plant. The very young leaf has predominantly acid wax. It would appear, therefore, that the cellular fatty acids condense to form the high molecular weight fatty acids. Reduction of these acids forms the alcohols; esterification forms the wax esters (57).

The cutin consists of polymerized dicarboxylic and hydroxy carboxylic acids (86). As cutin makes up the framework of the cuticle, its polar properties will make it swell when in contact with water. This increases permeability. The skins of tomatoes and grapes are known to be most permeable when in contact with liquid water (49). Turgid plants absorb 2,4-D better than do dry plants (118).

The outermost portion of the cutin layer is probably more highly oxidized and polymerized than the bulk of the layer. In addition it may be highly impregnated with wax molecules. This is in contrast to the inner layers which contact the cell wall and which are more polar (75). In the cuticle, then, there appears to be a gradient from low polarity on the exterior to relatively high polarity in the layers bordering the epidermal cell wall.

Surface chemistry of the cuticle.—In all cases penetration is controlled ultimately by adhesion between molecules (1). The adhesion between the solid cuticle and a liquid applied to it may be measured in terms of the contact angle (Fig. 1). The degree of spreading is also characterized by the contact angle. Thus the contact angle of a drop of clean water on a fresh *Sinapis* leaf is 96°, and that on a *Triticum* leaf 140° (36). This confirms the well known fact that a mustard leaf is easier to wet than a wheat leaf, a fact that has been utilized for removing wild mustard from wheat fields by spraying with aqueous solutions of dinitrophenol weed killers.

The magnitude of the contact angle and thus the degree of spreading are determined by three properties of the solid surface: (*a*) the nature of the chemical groups at the surface, (*b*) its degree of roughness, and (*c*) the presence or absence of air film below the drop (36).

Polar groups are capable of binding water through hydrogen bonds, while hydrocarbon chains only attract water through the much weaker van der Waals' forces. For this reason the contact angle between water and the waxy component of cuticle will be larger than between water and the cutin com-

ponent of cuticle. The outside surface of leek (*Allium*) leaves is so waxy that it gave a contact angle as on pure paraffin wax (59). When the fatty components were removed from a *Sinapis* leaf by extraction, the contact angle fell from 96° to 29° (36).

Contact angles below 90° are decreased by increasing the degree of roughness of the surface. If air film is present below the drop, the observed contact angles are greater than the true contact angles. The presence of the tiny waxy rods on the surface of leaves probably facilitates the trapping of air below drops of spray solutions (Fig. 1). The contact angle in such cases is large, and the drop will not spread. This is overcome by the addition of a suitable surfactant.

Penetration into leaves.—The waxy, unbroken cuticle of a mature leaf is very difficult to penetrate. Numerous experiments with the isolated cuticle from mature apricot leaves demonstrated that both organic and inorganic materials either did not penetrate or penetrated very slowly (75). Oils likewise failed to penetrate the intact upper leaf cuticle of apricot (110). It would seem then that the thick cuticle of mature leaves is an unlikely site for the penetration of chemicals applied in sprays.

The following alternative pathways for penetration remain: penetration through modified epidermal cells which overlie the veins; stomatal penetration; penetration through cracks in the cuticle; penetration of a cuticle stretched by expansion of underlying components or tissues; and, of course, penetration by a combination of one or more of these items. A brief discussion of these possibilities follows.

When the epidermis is stripped from a leaf, it can be observed that pure water does not wet the walls of the mesophyll (63). The addition of a surface-active agent will wet these walls. Pure water, however, does adhere to the surface of the vein and is soon absorbed into it. The surface of the vein is composed of a special type of parenchyma tissue, the bundle sheath (33). The cells of this tissue are thin-walled. Often cells similar to those of the bundle sheath extend from the vein to the upper or both upper and lower epidermis. These cells are concerned with conduction. Potassium ferrocyanide solution introduced into the leaves was found to move out quickly through the veins via the bundle sheath extensions into the epidermis. The bundle sheaths, their extensions, and the epidermis together constitute a supplementary conducting system in leaves (33, 124a).

It is quite possible therefore that aqueous solutions placed on the elongate epidermal cells above a vein, will be absorbed by this bundle sheath system and will arrive ultimately in the conducting elements of the vein proper.

Pure water probably does not penetrate stomata (116). Solutions with surfactants probably do. The latter, after having spread through the intercellular spaces of the leaf, will also arrive at the bundle sheath cells. They will do this either directly or via the mesophyll cells.

Since the wax components are not elastic as is the spongy cutin framework, it is evident that swelling of the cutin by water will spread the wax

components farther apart. This will have the effect of increasing the permeability of the cuticle. A similar effect is achieved if the epidermal cells and other underlying tissues are highly turgid. On the other hand when the cutin or the underlying tissue has a low water content, it will shrink and pull the wax units closer together. This decreases cuticle permeability. The sum total is a valve effect with considerable importance for the water balance of a leaf. When the cuticle is dry, this valve effect restricts water loss. When the cuticle is wet (such as with dew), the valve system allows water uptake. Similarly, chemicals will better penetrate the water-saturated cuticle (49, 118).

Structure and properties of the plasma membrane.—The protoplast does not lie as a smooth layer against the inside of the cell wall but penetrates it with a multitude of thin strands. Plasmodesmata are such strands which interconnect all living cells, making the protoplast of the plant body an organic whole (26, 33, 89, 92, 93). The outer walls of the epidermis also appear to have thousands of these plasmatic tentacles that extend until immediately under the cuticle (60 and Fig. 1). It has been claimed that these rhythmically extend and contract. In old foliage, ready to abscise, these structures disappear completely (60).

The outer surface of the protoplast, and therefore also the plasmodesmata, are lined with a very thin, yet relatively impermeable membrane, the plasma membrane. This membrane is another barrier to a chemical invading the living system of the plant. The plasma membrane is basically a lipoid layer 50Å thick with protein adsorbed on both sides. The protein layers of this sandwich structure are each about 70Å thick (29). A function of the protein must be that of stabilizing the lipoid layer; its effect on permeation will be discussed below under "pH effects."

Penetration of a chemical from an aqueous solution through the lipoid layer takes place in three steps: (*a*) Entry into the membrane. This involves breaking the hydrogen bonds that link the molecule to water, the overcoming of van der Waals' forces, and the formation of a hole in the membrane. The formation of the "hole" between the lipoid molecules is a result of thermal agitation (114). The chemical slips into this hole. (*b*) Diffusion through the membrane. This involves overcoming of the van der Waals' forces between hydrocarbon chains and between hydrocarbon chains and the diffusing molecule. (*c*) Exit from the membrane, which is the same as (*a*) but in reverse.

Studies on the rate of evaporation of water through fatty acid monolayers (7) are highly instructive for understanding of permeability phenonema. They demonstrate that the resistance to permeation is dependent upon the thickness of the lipoid layer. The logarithm of resistance to permeation is a linear function of the chain length of the molecules of the monolayer. Further, the presence of an aromatic solvent in the monolayer greatly increases permeability of the layer. This is in agreement with the theory of the action of phytotoxic oils on plants (110). This theory holds that the lethal effect of hydrocarbons is attributable to their solubilizing between the lipoid mole-

cules of the plasma membrane, thereby causing an abnormal increase in permeability.

If particles inside the protoplast are to be penetrated, the chemical must penetrate the membranes that surround them. These, however, do not necessarily have the same structure as the plasma membrane. Nuclei have an envelope which is a protein meshwork (6). Chloroplasts appear to have a membrane (64, 124). Nuclear and chloroplast membranes are believed to be perforate structures (91a). Mitochondria have a double membrane (79, 97). The endoplasmic reticulum is a closed submicroscopic vascular system, and therefore has a membrane (79a).

Uptake of Auxins

Adsorption.—In recent years evidence has accumulated that uptake of auxins is a two-step process. The first step is rapid and physical, and probably is simple adsorption. The second step is slow, steady, more complicated and appears to be metabolic. This sequence has been demonstrated for the uptake of indoleacetic acid from solutions by pea stem sections and by carrot tissue slices (82). The uptake of inorganic salts by potato tissue also starts off with adsorption (47). *Avena* coleoptile sections completed the rapid uptake of radioactive 2,4-dichlorophenoxyacetic acid in 30 min. (55). This was followed by a slow steady uptake, lasting at least 3 hr. Metabolic poisons had little influence on the first rapid process, but inhibited the continuous slow process. *Lemna* leaves floating on a solution with radioactive 2,4-D demonstrated the rapid initial uptake (16). The uptake of mineral salts such as phosphate by apple leaves follows a similar pattern (35).

There is little doubt that 2,4-D applied as an aqueous spray to weeds in the field also undergoes a rapid adsorption before it is taken into the interior. This adsorption explains the extraordinary rate at which 2,4-D is bound to weeds, a fact which surprised the early users of 2,4-D herbicide (109, pp. 203–4).

pH effect.—Sorption of radioactive 2,4-D by isolated cuticle of apricot leaves was found to follow a pattern similar to that of acid dyes (75). Both were highly pH-dependent. Sorption is high at low pH and practically nonexistent at pH values above six. This agrees in principle with the adsorption of IA by pea epicotyl sections and by carrot tissue slices (82), which was high at pH 4 and dropped to a very low value at pH 8.

It should be pointed out, however, that the sorption of 2,4-D on isolated apricot cuticle may not have a direct bearing on the uptake of 2,4-D by intact leaves or sections. First, the mature apricot leaf takes up 2,4-D very poorly through its upper surface. Second, no radioactive 2,4-D is sorbed by isolated cuticle at pH 9, yet at this pH there is a definite uptake of 2,4-D by foliage from sprays. Third, the absorption of auxins by pea stem sections, *Avena* coleoptiles, and particularly tissue slices, probably involves the plasma membrane primarily. In tissue slices no external cuticle is involved at all. Both plasma membrane and cuticle have fatty components as well as semi-

XUM

polar structures. For this reason it is logical that there should be a certain similarity in their sorption pattern.

Increased sorption of 2,4-D through increased H-ion concentration has been reported by many in the literature (42, 43, 17). The older explanation of this acid effect was that at a low pH molecules of weak acids such as 2,4-D and IA are to a large degree in the undissociated form. This form is more likely to partition into fatty phases, such as wax and the lipoids of the plasma membrane, than is the ionized form. It is a fact that there is an increasing partitioning of IA into ether from water as the pH decreases (38a). Approximately 50 percent of the total possible IA going into ether is achieved at a pH of about four, which is close to the pK of IA. This pK is 4.55 (110a).

On the other hand, it has been argued (20) that at a pH above four a compound such as 2-methyl-4-chlorophenoxyacetic acid is predominantly ionic, yet is highly active. Therefore, the pH effect at physiological acidities must be largely an effect on the plant rather than on the penetrating chemical.

The surface of both cuticle and plasma membrane contains free acid groups. In the cuticle these groups are from long chain aliphatic acids; in the protein portion of the plasma membrane these are amino acid residues. The lipid layer also probably contains acids. All of these are weak acids with a pK higher than five, higher than that of the penetrating regulators. For instance, at a pH of five the "surface acids" are largely undissociated and therefore the surface becomes more permeable to anions than at a neutral pH where the "surface acids" are largely dissociated and thus negatively charged. It was shown that permeability of lipoprotein monolayers to organic acid anions will increase if these layers become less negatively charged (20). Increased H-ion concentration should therefore increase the permeability of protoplasmic membranes to auxin ions. Similarly increased H-ion concentration will reduce the negativity of the acid groupings of cutin, thus explaining the increased adsorption of acid dyes and of 2,4-D on isolated cuticle.

In water culture, nitrate is taken up better by the root at a lowered H-ion concentration (108). This again may be due to an effect of acidity on the absorbing surface of the plant similar to that discussed above. If anions of regulators were supplied in the water culture, they too would be expected to be taken up better at a low acidity than at a higher one.

Wetting agents.—The increased spreading and wetting by surfactants are undoubtedly important reasons for the increased sorption and physiological activity of 2,4-D sprays containing detergents (19, 68, 91). Considerable differences were found between surfactants when their action was studied on the isolated cuticle (75). Cationic detergents such as Amine 220 allowed sorption of 2,4-D at pH 9, which never occurred without the detergent. The explanation again appears to hinge on a reduction of the negativity of the surface groups of cutin—this time not by acidity, but by the positive charges of the Amine 220 molecules sorbed at the surface.

Temperature effect.—Uptake is temperature dependent. Adsorption, the

first phase of uptake, is very little affected by temperature in the range encountered in biology. The increased thermal agitation at increased temperature will tend to decrease adsorption. Permeation through layers of oriented fatty molecules is highly temperature dependent. Many puzzling temperature effects on biological objects become clear when these are interpreted as effects of temperature on the plasma membrane. In maize roots the uptake of nitrate has a Q_{10} of 1.7 in the range between 20 and 40°C. But in the range between 5 and 10°C. the Q_{10} increases sharply to values as high as 7.0. This change was attributed to the increased viscosity of the fatty substances of the plasma membrane which are nearing solidification at these low temperatures (108).

Another illustration of this explanation of the effect of temperature on permeation, is found in insect cuticle. Some insect cuticles have closely packed, oriented wax molecules which are responsible for the very high resistance of the cuticles to water vapor (9). At a temperature just above the melting point of the wax there is a sudden increase in water permeability. It is at that temperature that the wax molecules become disoriented.

The general pattern is therefore clear: fatty molecules oriented in layers as micelles have a very low permeability when in the solid state. In the liquid state they have a higher permeability. When totally disorganized, as at lethal temperatures, such layers can no longer serve as permeation barriers. This temperature effect is on the barriers themselves, hence the principles discussed here must hold for any material, regulator or otherwise, penetrating through the plasma membrane or any other structure composed of fatty molecules in micellar orientation.

Molecular structure.—Penetration of molecules is much dependent upon their molecular structure. When penetration into and through fatty phases is involved, a less polar compound has a better chance to penetrate than a more polar compound. Thus increased penetration has been given as a reason for the greater auxin activity of the nitrile as compared to the more polar acid forms of IA and 2,4-D (13). This is even more striking if one remembers that the nitriles as such have no auxin activity and have to be converted inside the cells to indoleacetic acid and 2,4-dichlorophenoxyacetic acid.

For a similar reason hydrolyzable esters of biologically active weak acids are used in overcoming permeability difficulties (10). Ester forms of 2,4-D and related compounds such as MCPA, and 2,4,5-trichlorophenoxyacetic acid have been used successfully as herbicides. Aliphatic esters penetrate the cuticle, but do not seem to move out of it into the vascular system (25). When such esters are used as a broadcast spray for brush control, several pounds per acre may be lost in the thousands of pounds of cuticular lipoid of the vegetation. Heavy esters formed by alcohols that have both water- and fat-solubility, are proving effective as translocated herbicides. Thus the butoxyethanol esters of 2,4-D and 2,4,5-T are effective in this respect (25).

Until recently it was thought that the *ortho* position of the ring of auxin molecules was essential as a point of attachment in a reaction leading to auxin activity (71a). It is now known that the amide form of 2,6-dichlorophenoxyacetic acid has auxin activity which the acid form does not have. Moreover, the addition of a methyl, ethyl, or propyl radical to the *alpha* carbon of the side chain of 2,6-dichlorophenoxyacetic acid also causes the acid to become active as an auxin (76, 77). It seems, therefore, that increasing the lipoid solubility is all that is necessary to demonstrate auxin activity in this type of molecule. From this it follows that the 2,6-acid does not show auxin activity because it does not penetrate sufficiently. Further, it has been suggested that anti-auxin activity of compounds may be due to interference with the uptake of auxin rather than solely by competitive inhibition at a growth center (8, 50).

The need for a proper balance in the molecule between lipophilic and hydrophilic groupings as an essential requirement for auxin activity has been recognized for many years (112). This requirement was reconfirmed recently (18). Facilitated penetration into cells by lipophilic radicals was also demonstrated for anti-auxin and defoliant activity of a series of maleimides (111). The presence of a lipophilic tail on a molecule is also required for fungicides to penetrate spores (48, 84, 85). It may make the difference between a commercially successful biocide and a failure. This was clearly demonstrated for the bactericide hexylresorcinol; the hexyl tail made the difference (48). A final example of the importance of oil-soluble properties is the penetration of compounds into the rabbit skin. Penetration is nearly a straight function of the ether/water partition coefficient (106). The more of a compound which partitions into the ether, the more it penetrates the animal skin.

Partitioning properties of a molecule between a semi-polar lipoid and water are indeed a reasonable guide for penetration into cells (23). However there is a significant difference between a molecule dissolving in an oil solution and a molecule solubilizing into a plasma membrane. In the oil solution the molecules are randomly distributed, while in the plasma membrane the lipoid molecules are arranged in micelles. This may explain why one molecule may penetrate an organism, whereas a closely related molecule, having the same partition coefficient, may fail to do so. An example of this type may be N-*n*-octyl thiourea, and N-*tert*-octyl thiourea. The first one is a good fungicide, the second one is inactive (85).

Partitioning of compounds into fatty phases of the cell involves not only the plasma membrane, but also the other fatty bodies such as mitochondria and chloroplasts. These structures undoubtedly have their fatty molecules arranged in micelles.

Penetration.—There is probably no stomatal penetration by plain aqueous solutions of auxins such as 2,4-D (116), IA, and naphthaleneacetic acid—that is, solutions without detergent, and at an acidity not too far removed from neutral. In leaves, penetration of such solutions must involve

cuticle and epidermal cells. It is possible that there are areas of preferred penetration and that these are located above the veins and bundle sheaths.

In formulations that contain surfactants, spreading is much improved. Air films between solution and cuticle no longer hinder penetration. It is probable that stomatal penetration takes place. The solution then spreads along the intercellular spaces and wets the walls of mesophyll and bundle sheath cells. Penetration through cracks in the cuticle is improved. A better contact between solution and cuticle should also facilitate penetration between the wax particles of a cuticle stretched by underlying turgid cells. Ultimately, the auxins arrive in the vascular system of the leaf where they will be transported according to principles discussed below.

TRANSLOCATION

Pattern of distribution.—The translocation of regulators follows a pattern similar to that of most other organic compounds, and even some inorganic compounds. An exception is the translocation of the naturally occurring growth regulators, the hormones, which will be dealt with in the next section. In general, one may say that if a compound is taken up by the leaves, it is transported out of that leaf via the phloem. When a compound is taken up by the roots, transport upward into the aerial portions of the plant takes place through the xylem. Widely different materials such as phosphates (14) and indoleacetic acid (98) can move up through the xylem into the leaves, and from there they may be rerouted via the phloem. Since some of this involves downward movement, it is possible that materials circulate through the plant.

When radioactive Schradan[2] and radioactive phosphoric acid were given to the root system of citrus seedlings, it was found that the two were taken up and distributed in exactly the same manner (65), following the pattern indicated above. 2,4-D also behaves this way (67).

When a material is applied to the leaf, it is invariably found that the lower epidermis absorbs more than the upper. This is true for 2,4-D (118), for systemic insecticides (11), and for urea (24). There are three reasons for this: a larger number of stomata, a thinner cuticle, and the presence of the phloem on the lower side of many leaves. Young leaves absorb material more readily than older ones. This holds for 2,4-D (34, 118), Schradan (11), and phosphoric acid (35). With sprays of inorganic nutrients it was found that most of the material that sticks to the leaf is absorbed, and that most absorption occurs when the deposit appears to be dry (103, 104). It would seem that a similar situation holds for 2,4-D.

Since transport of regulators and of phosphates have so much in common, it may be well to look at some further features of phosphate transport. The first step in phosphate uptake by roots is adsorption (41). Apparently, roots are not capable of absorbing phosphate for transport over their entire length. The root tip accumulates large amounts of ions, but these are not transported because the xylem is not yet functional (122). The region 3 cm.

XUM

back from the root tip of barley absorbs water and solvents best for the purpose of further translocation (122). For *Vicia* roots it has been shown that phosphate is first translocated throughout the root system, even against the direction of normal water flow, before it enters the shoot (123).

Rate of movement.—Movement of 2,4-D from the outside of the epidermis into the phloem is a slow process; a rate of 30 microns per hour has been recorded in bean leaves (30). Once within the phloem, 2,4-D moves with speeds between 10 and 100 cm. per hour (30). These values are in general agreement with more recent findings with radioactive 2,4-D.

Just as the pattern of distribution of regulators is like that of other compounds, so there is a similarity in rates of transport. When the products of photosynthesis were followed after administration of $C^{14}O_2$ (113), it was found that sugar was translocated through soybean stems at a rate of 85 cm. per hour, a figure well within the range of that found for 2,4-D. Chilling of the stem caused a drastic reduction in phloem translocation (100, 113), but not in xylem transport. Recent reports from Russia (4) indicate that in sugar beets and pumpkins the products of photosynthesis move toward the root at a rate of 70–100 cm. per hour, and toward the fruit and growing shoot at 40–60 cm. per hour.

Though these figures may suggest that all materials move through the phloem at equal rates by a mass flow, evidence is accumulating against this simple and convenient concept. Thus, sucrose is translocated faster than fructose and glucose (113). Foliar applications of radioactive potassium, phosphorus and cesium salts indicate that they move through the phloem at different rates (100). Studies with P^{32} and tritiated water show that while water moved at 180 cm. per hour through the phloem, the phosphate at the same time covered only 60 cm. per hour (15). The picture emerging is not that of a mass flow but rather of a chromatogram in which the solvent front moves ahead of the solutes.

Recent protoplasmic studies of the phloem (28) as well as electron microscope studies of the sieve plates (46, 93) leave the validity of the concept of mass flow in doubt. Continuity of the cytoplasm rather than continuity of the vacuole is indicated. Even though no concise mechanism for phloem transport can be suggested, it is still within the realm of possibility that spreading along an interface is involved. There must be many such surfaces in cytoplasm. While spreading along a solid surface would be too slow, spreading along a liquid-liquid interface, or even a semi-liquid interface may well be possible. Spreading along liquid-liquid interfaces is very rapid; rates of 20 cm. per second have been observed (2). In spreading along liquid surfaces the molecules of the underlying liquid move along the surface with the spreading substance. Obviously this is not so in spreading along solid surfaces. Several years ago such a liquid-liquid system was suggested for movement of compounds through plants (107).

Effect of carbohydrates.—Movement of 2,4-D does not take place out of leaves which are low in carbohydrates (66). Sugars, provided either through

photosynthesis or through application, restore translocation (90). Sucrose was most effective, followed by glucose (54). Applied glucose-phosphate was not effective, perhaps because it could not penetrate the leaf. Leaf carbohydrates are necessary for translocation, but not for the absorption of 2,4-D (44). The necessity of leaf carbohydrates is not specific for 2,4-D transport, since it would appear that a similar relation holds for translocation of Schradan (102).

Increased translocation of 2,4-D has been induced by boron (69). It would seem that this is an indirect effect, as boron is known to promote translocation of sugar (37, 38). Although the relation between sugar and translocation of 2,4-D and other material has been interpreted as supporting the mass-flow theory, there is a contention (45) that the carbohydrate is required to supply energy for the 2,4-D transport from the leaves, rather than to activate a mass flow mechanism.

Translocation through intercellular spaces.—When a drop of 2,4-D dissolved in kerosene was placed on a leaf, 2,4-D was found to move out of the leaf into the stem, even when the plant was in the dark and regardless of carbohydrate content (83). The explanation is simple. The kerosene moved through the stomata and spread via the intercellular spaces throughout the plant. It is well established that oils move through plants via the intercellular system and not the vascular system (110). Movement of regulators and other materials in the vapor phase must also occur through the system of intercellular spaces. Volatile esters of 2,4-D undoubtedly could be distributed via this system, as diffusion through the gas phase is very rapid.

Molecular structure.—Just as the uptake of compounds is dependent upon molecular configuration, so is translocation. The introduction of a methoxy group in the *alpha* carbon of the side chain of 2,4-D improves its translocation. A similar improvement in translocation was achieved by the introduction of the "lactic acid" group in carbamates (70, 80). No exhaustive studies have been made and the effects are not yet understood.

Polar Transport of Hormones

Occurrence.—Apex-to-base transport in parenchyma, without the benefit of a vascular system, appears to be a rather unique property of natural auxins. It sets these compounds apart from synthetic auxins. However, it is doubtful that the naturally occurring auxins are the only materials showing polar transport. One will recall that polar movement of fluorescein in hair cells of *Cucurbita* was demonstrated (120, p. 102).

This property of polar transport has been shown for indoleacetic acid as well as for indoleacetonitrile (12) in *Avena* coleoptiles. Indolebutyric acid, anthraceneacetic acid and naphthaleneacetic acid show some evidence of being polarly transported in *Avena*, but much less than IA (121). Transport by the polar system is very slight for 2,4-D (101).

In hypocotyl sections growing under aseptic conditions IA is absorbed polarly (31). Similarly, in *Helianthus* pith cylinders growing on a plate of

culture medium, low concentrations of naphthaleneacetic acid are taken up polarly (95).

Polar transport is also found in woody stems; it occurs in the living cells of both bark and wood (78). In leaves auxin moves through the veins (120, 125). When IA is applied to roots, it is rapidly absorbed and moves upward with the transpiration stream. It may move laterally into the tissues surrounding the xylem and then is rerouted in a basal direction (98). In squash and tomato the rate of upward movement was found to be 70 cm. per hour, and in the basal direction 20 cm. per hour. The latter evidently is phloem transport, as much lower values are found for transport in parenchyma where movements are of the order of only 1.5 cm. per hour (120).

It is probable therefore that physiological concentrations of natural auxins and a few synthetic ones move in the parenchyma only in a basipetal direction. When they are applied at higher concentrations, they penetrate into the vascular system and then follow whatever stream is moving there (115). In the transpiration stream movement will be upwards.

In *Coleus* the advent of flowering decreases the basipetal polarity of the stem (62). However, polarity in this plant is already weak in the vegetative stem (51, 52), probably because transport of auxin here is dependent upon vascular strands (53).

By a simple and ingenious technique the rate of transport of native auxin in woody shoots of apple was recently determined (40). A typical polar auxin movement was found. It has long been held that the amount of auxin transported, but not the rate of auxin transport, is influenced by temperature (120). It is now apparent (40) that both rate and amount transported are equally temperature dependent. The original idea resulted from a misinterpretation of data. The rate of transport of native auxin is almost nil at 0°C. and reaches a value of about 1 cm. per hour near the optimum temperature of 27°C. In the range between 10 and 30°C. the transport has a Q_{10} of the order of two. The transport of native auxin is further dependent upon aerobic conditions. The rate of auxin transport in the apple shoots was found to be proportional to the oxygen concentration (40).

The reviewer has seen no successful experiments in which the rate of transport of indoleacetic acid was measured by radioactive tracers. This is in contrast to 2,4-D which is widely and successfully used with C^{14} substituted in at least three different portions of the molecule. The reason for the lack of success with radioactive IA is not due to lack of effort, but due to the extraordinary lability of IA in plant tissue. At the cut surface of tissue, this enzymatic destruction can be counteracted by small amounts of cyanide (99). Indoleacetic acid is destroyed by a variety of enzyme systems (39, 94, 105), in addition to the well known indoleacetic acid oxidase (96). The latter is an inductive enzyme system. One may wonder how native IA in the plant escapes destruction. It could well be that a combination with protein affords this protection. It is well known that IA combines readily with protein.

Nature of polar transport.—In searching for a possible mode of action of the polar transport system, one has to consider two aspects: transport itself and its polar nature. Auxin transport through parenchyma is 200 times faster than diffusion. This has been known from the earliest time that auxin was isolated from plants (119). It was believed at that time that protoplasmic streaming could account for this relatively high rate. This idea was further expanded (32) in the thought that the site of polarity was in the transverse membranes of the parenchyma. The effect of respiratory poisons on auxin transport was thus explained in terms of inhibited protoplasmic streaming.

However, there is evidence (22) not too well known, which would seem to make the protoplasmic streaming theory untenable. Saponin applied to coleoptile sections could stop protoplasmic streaming of *Avena* coleoptile cells without interfering with the transport of IA. Sodium glycocholate at low concentrations abolished polar auxin transport without interfering with protoplasmic streaming. Sodium glycocholate is a bile substance which is surface active and has been shown to increase plasma membrane permeability. These results suggest that polar auxin transport does not involve protoplasmic streaming, but involves plasma membranes and surfaces. Spreading may well be along liquid-liquid interfaces (107) while polarity might well be explained by a pH gradient.

Triiodobenzoic acid modifies the response of plants to auxin in such a way as to appear as if this acid abolishes the polar auxin transport (58, 72, 73). Since it has been shown that TIBA is a rather strong—SH reagent (61), one wonders if the polar transport system is sensitive to —SH reagents in general. If that should turn out to be the case, anti-auxin effects like defoliation by well recognized —SH reagents such as the maleimides (111), might also be due to inhibition of normal auxin transport. Such a view would shed a new light on the auxin gradient theories for abscission, which have recently been proposed (3, 53). Interference with the movement of auxin in roots by 2,4,6-trichlorophenoxyacetic acid was recently suggested (20a). The reason was that this anti-auxin prevented geotropic curvature at concentrations that did not alter growth in length.

Conclusion

Absorption and translocation of regulators follow the principles of organic compounds in general.

There are two main barriers to penetration, the cuticle and the plasma membrane. The cuticle is a sponge-like structure of semi-polar cutin with wax platelets in the holes and tiny wax rods on the external surface. Cuticle of some sort covers all (primary) plant surfaces exposed to air, whether outside or inside the leaf. Stomatal penetration makes the internal cuticle available for absorption. The plasma membrane has a sandwich-like structure: closely packed fatty molecules between protein layers. It covers all surfaces of the protoplast, probably even the fine strands that penetrate cell walls.

The first step in the uptake of auxins is physical: adsorption. A final step is metabolic in nature. Wetting agents, a low pH, an oil/water partition coefficient in the direction of oil are all factors that influence uptake of auxins favorably. Other regulators follow the same principles.

Regulators that penetrate the leaf are translocated via the phloem. Regulators that move out of the root into the aerial parts do so via the xylem. Auxins may move up in the xylem and back down the phloem. Native auxins display a striking, one-way apex-to-base transport through parenchyma.

An attempt has been made to make a unified picture out of the many isolated facts that underlie the absorption and translocation of the many organic compounds that plant physiologists spray on plants.

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XUM

EFFECTS OF LIGHT QUALITY ON PLANT GROWTH[1]

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Introduction

The external appearance of plants growing under natural conditions of environment is determined by genetic and physiological factors. The genetic factors set certain limits to the variation in the relative and absolute measure of the individuals within one species. Within these limits, physiological factors determine the formative aspect. Several investigations have shown that the genetic boundaries are wider than might be assumed, provided that the physiological treatment is adequately differentiated.

Among the physiological factors light is a very important one. Light not only supplies the energy upon which plant life is based, but via various photomorphogenetic mechanisms it also plays an important role in directing this energy along the various possible metabolic pathways.

The effects of light quality on plant life are manifold; studies on this subject by plant physiologists are numerous and extend over more than 150 years. The efforts of Senebier, e.g., have lived on in the "Senebier domes," double walled glass covers to be put over plants as spectral filters in which the space between the double walls was filled with coloured fluids; they have been used, e.g., by Sachs (1).

Since for most processes other than photosynthesis, the amount of light energy initially absorbed is low in relation to the over-all effect, these light reactions can be considered to belong to the group of photo-stimulus processes (2) which are characterized by a fairly complicated dose-effect relationship, and which are exothermic in that they ultimately release or direct an amount of stored energy which may be very large as compared with the energy content of the light responsible for the stimulus.

Effects of light quality on various phases of growth and development have been studied in almost every major group of plants. A complete evaluation of all the material available from the viewpoint of our present knowledge, including the extensive development that has taken place during the last decade, would be interesting, but is far beyond the scope of this article.

The present discussion will be restricted mainly to investigations on Cormophytes, especially Angiosperms, and will cover especially work on formative effects: elongation of stems, development and shape of leaves, and, finally, root growth. The spectral sensitivity of photosynthesis will not

[1] The survey of the literature pertaining to this review was concluded in October, 1955.

[2] Communication No. 142 of this laboratory.

be discussed in detail; only a few remarks of general bearing on this subject will be made (see pages 388–89).

We will also refrain from discussing phototropic phenomena, photoperiodic reactions *sensu stricto* (the initiation of flowers), and seed germination. These subjects will be touched upon only when recent results are of importance for reasons of comparison and may contribute to an understanding of the effects of light quality on stem and leaf growth. Various reasons have led to the choice outlined: First, a restriction of the field seems unavoidable in favour of a more elaborate discussion of some recent findings; Secondly, the phenomena especially related to flower initiation and seed germination have been extensively reviewed recently [Parker & Borthwick (3); Borthwick *et al.* (4); Lang (5, 6, 7); Liverman (8)]; Thirdly, we feel that the formative reactions observable in stem elongation and leaf growth are simpler and more direct expressions of the same class of light effects to which photoperiodic effects (and probably certain photobiological phenomena with respect to seed germination) belong.

Attention may be drawn to some older reviews on "light and plant growth" which give special attention to the spectral effects. The extensive article by Burkholder (9) covers all imaginable subjects. His chapter on "light quality" (pp. 120–23) in which the work of the Boyce Thompson Institute predominates is of special interest in connection with the present paper.

A comparable study was published in Dutch by Funke (10). It contains a discussion on formative effects of light, etiolation (of green and nongreen plants), effects of light on seed germination, on the structure of leaves, and on photoperiodism, including experiments by the author.[3] Roodenburg (11) surveyed literature on the effects of additional or exclusive illumination from various light sources (neon, sodium, mercury lamps, etc.) [see also (12)]. Åberg (13) elaborately discussed "photomorphotic" phenomena from physiological and ecological viewpoints, together with his own experiments. Some of his findings and conclusions are still of interest, e.g., his statement that short infrared radiation (passing through 3 cm. water) probably favors internode elongation in the tomato plant. Åberg concluded further that "when only the small quantity of light necessary to prevent the most pronounced etiolative response" is given, the region of long wave lengths of visible light is most effective, while, in light of higher intensity, especially the shorter wave lengths will retard stem elongation in seedlings.

The extensive experiments on effects of light intensity and spectral quality on plant growth made during 20 years at the Boyce Thompson

[3] Funke, in the years just prior to his early and unexpected death in 1946, produced some quite remarkable survey books on plant physiological subjects in the Dutch language. They are original in the choice of their subjects, the manner of presentation, and the inclusion of the author's unpublished work (*Experimental Plant Sociology*, 1943; *Formative effects of light*, 1944; *Water plants*, published after Funke's death, 1950).

XUM

Institute by Arthur, Popp, Shirley, and their co-workers have been summarized by Crocker (14). The conclusions from this work, relatively advanced in an early period, agree well with more recent data. It was demonstrated that the blue-violet end of the solar spectrum is required for sturdy growth and for flat expansion of the leaves. If all radiation is above the wave length of 529 mμ, the plants are spindly, elongated, and the leaves curled and epinastically curved. If radiation is above 472 mμ, the appearance of the plants is intermediate between that obtained with the full daylight spectrum and that obtained in the wave length region above 529 mμ. It was shown that light intensity was not a decisive factor in this respect.

Before starting a discussion of recent experiments, we wish to make two more general remarks, viz., on "etiolation," and on "critical day-length" in relation to photoperiodic phenomena.

Plants grown in darkness or at very low light intensities usually lack chlorophyll, show excessive elongation of stems, and reduction of leaf size and leaf development. The appearance of such plants is normally described as "etiolated." The above-mentioned aspects of "etiolation" are not necessarily all present. Under special conditions plants may be produced with fairly normal appearance, but with very little chlorophyll [15, 16, 17, cf. also Funke (10)], while some of the above aspects may arise in "green" plants owing to certain combinations of spectral irradiation (see pages 378–379).

Moreover, the "etiolated" appearance generally has been associated with low available light energy, thus being a kind of negative effect. Recently, however, evidence has favoured the view that the etiolated appearance may be produced by certain irradiation patterns, which supply special spectral regions in addition to others (see page 378). Therefore it seems useful to attach the terms "etiolated," "etiolation," and "etiolated appearance" only to plants really grown under low light conditions and not to certain morphological characteristics, if one wants to retain these terms. This usage will decrease confusion and promote further analysis of the underlying physiological reactions. Thus we will mean by "etiolated material" plants or plant organs produced at very low light intensities or in complete darkness, and, for reasons outlined below, we will discuss light reactions of such material separately from those of light grown plants.

In literature on photoperiodism many efforts have been made to establish the "critical day-length," i.e., the day-length that will just enable (or prevent) flowering. This usually has been determined for plants receiving a short day in white light (either daylight or artificial light) of high intensity, preferably supplemented by an adjacent period of low light intensity, or a short "night break" at high intensity. It has been considered by many workers that the critical day-length is rather fixed, notwithstanding its relatively slight dependence on certain other conditions [e.g. temperature (18)]. Recent data (see page 381), however, give evidence that the spectral composition of the light during the main light period may strongly influence

the critical day-length, so that even a long-day plant may fail to flower for extended periods in continuous light if this light lacks certain spectral regions. Such cases necessitate that a definition of the term "critical day-length" include a statement of the kind of light given during the main light period. In addition, it can be noted that a related phenomenon is evident in cases in which a short day of high light intensity is supplemented by a period of weak light or by a brief light flash in the middle of the night. It has been shown in such cases that the photoperiodic effect is dependent not only upon the amount of light (and, within a certain range, upon the duration of illumination) to which the plant is exposed, but also upon the spectral quality of the supplementary illumination [e.g., the work of Borthwick and his associates, cf. (3)].

There are several reasons for considering the spectral dependence of light reactions in etiolated and light-grown plants separately. Usually there is an appreciable difference in the absolute rate of growth in length, and, moreover, there is a difference in the spectral dependence of inhibition of elongation. In etiolated plant material the red spectral region is most effective in this respect, whereas in light-grown plants, and at high light intensities, the violet-blue region of the spectrum is especially effective. This difference in response is not necessarily associated with the presence or absence of chlorophyll (17), nor even with the capacity to synthesize chlorophyll (16).

Effects of Light Quality on Growth of Light-Grown Plants

The main progress in the period 1945–1950, chiefly considered in the previous review in this series (3), was the establishment of detailed and reliable action spectra for the photoperiodic behaviour by the authors of the mentioned review article, and their co-workers. Such action spectra were determined for two short-day plants and two long-day plants. The spectra were all of the same type, with minor mutual differences, showing a very pronounced maximum of activity in the red part of the spectrum between about 600 and 660 mμ. A tendency towards a second, smaller maximum in the blue-violet region was always indicated. No activity whatsoever in the near infrared was observed, or even considered. "There was a steep cut-off in the vicinity of 7200 Å." Speculations as to the active pigment drew attention to phycocyanin as a possibility, or to a similar chromoproteid.

The main development in the period elapsed since the review mentioned above may be characterized by the following rather startling discoveries: (*a*) The observation of the effect of near infrared radiation on various light-sensitive formative and developmental phenomena; (*b*) The evidence for antagonistic action between certain spectral regions; (*c*) The recognition that various physiologically unrelated phenomena have similar action spectra and are subject to similar antagonisms, especially between red and infrared radiations; (*d*) Observations leading to a more profound discussion of

the phenomena of "etiolation" and "critical day-length." We will discuss these points in some detail.

Up to about 1950, the only definitely established photobiological effect of near infrared irradiation in higher plants was that described by Flint & McAlister (19) who found germination of lettuce seed promoted by red and inhibited by near infrared radiation, with a maximum activity at about 730 mμ. Moreover, mostly upon rather indirect evidence, some authors had ascribed formative and photoperiodic effects to near infrared radiation [Åberg (13), Roodenburg (11), see also page 374].

The first clear-cut direct demonstration of a definite photobiological effect of infrared radiation in developed plants, together with additional rather unexpected effects of supplementary irradiation in the violet-blue region, came from experiments of the present authors and their co-workers (20). Recently this work has been discussed rather elaborately by Lang (6, 7) and by Nuernbergk (21). The early articles (20, 22, 23) seem to have been somewhat overlooked by Borthwick *et al.*, who, in their 1952 article (24) on infrared effects, refer only to private, more recent communications of Wassink and his associates.

Wassink & van der Scheer (22) describe the equipment with which the experiments on day-length extension with light of restricted spectral regions have been made. It consists of a series of cabinets providing illumination up to *ca.* 3,000 ergs/cm.²/ sec. in fairly narrow spectral regions, viz., violet, blue, green, yellow, red, and infrared. The illuminations are obtained from monophosphoric fluorescent tubes (violet, blue, green, red), sodium lamps (yellow), and incandescent lamps (infrared), combined with glass filters so as to obtain light from the desired spectral region only. The equipment allowed plants to be exposed in full, during considerable periods, in light of good spectral quality. Thus, it should provide some supplement to the well-known spectrograph of the Beltsville group [cf. (3)]. The first plant investigated was *Brassica Rapa*, f. *oleifera*, subf. *annua*, a crucifer supposed to belong to Funke's "group 4" (25, 26), in which flowering should be promoted especially by blue light, and not by red light. During the experiments (20), plants received a daily 10 hr. basic illumination in white light ("daylight" fluorescent tubes), and 8 hr. day-length extension in each spectral cabinet, with controls receiving either 8 hr. additional white light, or darkness. Flowering, indeed, proceeded according to Funke's scheme, yielding flowerstalks in white, violet, and blue light but not with day-length extensions in green, yellow, and red light. The plants which received no day-length extension ("dark controls") did not flower. Quite remarkably, the plants receiving a day-length extension with infrared showed the same behaviour as those in the blue, violet, or white cabinets. Moreover, it was found that, especially in the infrared, stem elongation was independent of flower bud development. The initiation of the inflorescence primordia was fairly early in all spectral regions, although there are differences in time; greater differences occur in the subsequent develop-

ment. The marked difference in reaction of the plants in red and infrared appeared the more striking because the red had a rather considerable amount of near infrared. As a possible suggestion, it was mentioned at that time that the action of the near infrared may be suppressed by red (20, p. 1472).

A subsequent paper (23) was especially concerned with internode elongation in various plants upon day-length extensions in the same spectral regions. The main results are: *Brassica Rapa*, f. *oleifera*, subf. *annua*, develops flowers in the blue, violet, and infrared cabinets also when a basic white light illumination period of only 8 hr. daily is extended by 4 hr. of irradiation at an intensity of 1,000 ergs/cm.^{2}/sec. Under these conditions the total daily illumination period is only 12 hr., and the white light control plant did not flower.

Stem elongation in blue, violet, and infrared radiation starts immediately and is absent in any of the other spectral day-length extensions, including white light extension. Of considerable interest is a series in which *Brassica* plants received a basic (high intensity) light period of only 5 hr. white light, supplemented with 4 hr. day-length extension in the various spectral regions. In an early stage, elongation is only observed in plants receiving violet, blue, or infrared supplementary irradiation. In all kinds of irradiation, flower primordia and flower buds were finally produced, even in the dark controls. However, only in the plants receiving violet, blue, or infrared supplementary irradiation, did larger flower buds develop normally. These buds, however, did not open, probably because the energy balance of the plants under these conditions was rather unfavourable. Plants in the (8+4 hr.) series receiving supplementary white radiation do not develop their flower buds, whereas flower buds are developed by plants in the (5+4 hr.) series receiving supplementary blue radiation; this fact was explained by assuming an antagonism between certain spectral regions. Since, after the fifth hour of each cycle, the (8+4 hr) "white" plants receive much more blue light (while receiving an additional irradiation of 3 hr. at high light intensity) than the (5+4 hr.) "blue" plants, the above observation "leads [one] to suppose that other parts of the spectrum have an antagonistic effect" [23 (p. 424)].

In the (10+8 hr.), (8+4 hr.) and (5+4 hr.) series marked stem elongation in *Brassica* was observed only upon supplementary irradiation with violet, blue, or infrared. It seems still more interesting that the same reaction for stem elongation was found in young plants of *Cosmos bipinnatus* in similar experiments [see e.g. (23), Fig. 2, and plate 4 A].

Very striking stem elongation with complete absence of any correlation with flowering was observed in *Lactuca* seedlings,[4] receiving a 10 hr. basic daily irradiation with white light, supplemented by the usual 8 hr. low intensity spectral and white day-length extensions; A. Plants receiving a day-length extension with violet, blue, or infrared radiation showed a very

[4] In (23) the variety used is erroneously quoted as "Meikoningin," this should be "Wonder van Voorburg."

marked elongation of both stem and leaves, so that a definitely "etiolated" appearance was produced [(23), plate 4 B]. Plants receiving darkness did not show this etiolated appearance, and neither did plants receiving supplementary white light, or irradiation from any of the other spectral regions. The elongations of stems and leaves show the same spectral relations [(23), Figs. 5 and 6].

We have discussed these early papers in some detail because, in some instances, they appear to have been overlooked, and they give rise to the following conclusions of general interest: (*a*) Flowering in *Brassica* depends on wave length in much the same way as Funke (26) has mentioned for certain plants, especially members of the Cruciferae. (*b*) Flowering in *Brassica* occurs also within short days provided a day-length extension within the short day is given in certain spectral regions, demonstrating that the "critical day-length" is spectrally sensitive. (*c*) A clear cut photobiological effect of infrared radiation on full-grown plants was demonstrated for the first time. (*d*) Certain formative effects in various plants (especially internode elongation in *Cosmos* and in *Lactuca* and leaf elongation in *Lactuca*) depend on the spectral quality of day-length extension in the same way that was observed for flowering in *Brassica*. These effects are completely independent of flower bud formation, and seem to be more basic than the photoperiodic reactions. (*e*) Especially in *Lactuca*, it is obvious that certain day-length extensions produce an "etiolated" appearance which can be ascribed to certain spectral qualities of the "light regime" (especially during day-length extension) rather than to lack of light. Especially the fact that extension of day-length by "darkness" causes a less etiolated appearance is important in this respect. Thus, the etiolated appearance is to be considered a "positive" effect of certain spectral properties of the day-length extension. (*f*) Certain observations lead one to conclude that the violet, blue, and infrared regions react antagonistically to the green, yellow, and red regions with respect to the formative effects discussed.

The above observations have now been extended (27 to 31) and, further, the formative effects of light of restricted spectral regions on the growth of plants under complete exclusion of "white light" have been studied (32, 33). Plants can be grown in especially constructed cabinets for long periods (several weeks) in light of narrow spectral regions of relatively high purity (cf. 32, 33) without any white light. They grow quite well and gain considerably in dry weight (31, 32). The light intensity in these cabinets is measured with a "spherical radiation meter" as described by Wassink & van der Scheer (34, 35). The light intensity in the various cabinets was kept on an equal erg-basis, since the more desirable equal quantum basis would imply an unfavourable drop of intensity in the long wave length compartments. For comparison a white light compartment (daylight fluorescent tubes) was included.

Most plants show short growth in white light (daylight fluorescent tubes) which is even more evident in blue and violet light. In green light, and still

more in yellow and red, plants show a marked stem elongation. In tomato (variety Vetomold 121), which was used for fairly extensive experiments, the plants in the yellow and red cabinets showed conspicuous epinasty of the midribs and lateral veins of the leaves, in addition to stem elongation. This is in strong contrast to the behaviour of plants belonging to the same variety, exposed to a short day in white light, supplemented by some hours of fairly low intensity radiation in narrow spectral regions (32). The effect of exposure to near infrared radiation has not yet been investigated adequately since no experiments so far have been made with near infrared radiation only (supplemented, e.g. by sugar feeding). A cabinet equipped with red glass filters and incandescent light contained as much red radiation (wave lengths less than 700 mμ) as the red cabinet, and, in addition, about three times the amount of near infrared radiation. In this cabinet the plants showed the features of the red cabinet in an exaggerated way, so that the effects of red and infrared seem to be much the same.

Stolwijk (31) has established that the dry weight of the plants in the violet, blue, green, yellow, and red cabinets shows a regular increase in the above-mentioned order, suggesting little spectral dependency of the efficiency of photosynthesis under the experimental conditions [cf. Rabideau, French & Holt (36); see also pages 388–94 but rather a reaction to the increased quantum content of equal energies towards the longer wave lengths The increase in dry weight in relation to wave length was mainly a result of increase in stem weight; roots and leaves showed smaller differences. This indicates a certain relative promotion of leaf area and root formation in the blue-violet region.

Fortanier (29) studied growth and behaviour of *Arachis hypogea* in these cabinets. Here, also, plants were very elongated in yellow and red light, moderate in green, short in blue and white. The same order of response was observed for the flower buds. In red and yellow light the flowers did not open, at least not at 20°C.; at 26°C. they were found to open in all spectral regions. (It was evident that the time of flower bud opening was determined three days before, by the moment at which illumination begins daily.)

Also leaf movement in *Arachis* showed a definite spectral sensitivity: the leaves remained closed in red and yellow light of both high and low intensities at 20° and 26°C. Leaf position was influenced also by the preceding illumination. Blue light has a strong after-effect on leaf opening; red light does not show an after-effect. In experiments with mixed blue and red illumination, the opening effect of blue light on leaf opening appeared to be stronger than the closing effect of red light; 100 ergs/cm.²/sec. of blue light were sufficient to cause the leaves to open at an illumination of 9,800 erg/cm.²/sec. of red light. In low light intensities the leaves showed specific reactions to the spectral regions only if they had had darkness or a small amount of light before; they then opened in blue, green, and perhaps in infrared. In blue light and under illumination from daylight fluorescent tubes the leaves opened more widely than in natural daylight especially at medium light intensities.

Spinach was found to react upon day-length extension at relatively low light intensities in a way different from that of various other plants (27). Plants were found to elongate and flower with day-length extensions in white (daylight fluorescent tubes), green, yellow, and red light, and remained in the rosette stage with day-length extensions in violet, blue, infrared, and darkness ("short day" treatment).

Some remarkable results, though still of a somewhat preliminary nature, have been obtained with *Hyoscyamus niger* [Stolwijk & Zeevaart (37)]. In long days (16 hr. or 24 hr.) in the high intensity cabinets, rapid flowering was observed in the blue and violet compartments, and in the red, provided some 30 to 300 per cent infrared radiation (from incandescent lamps, filtered through the same red glass) was admixed. Also in white light (daylight fluorescent tubes) flowering was obtained, somewhat less rapidly, however, than in the above. Plants in yellow, red, and especially green remained vegetative. Red showed some stem elongation after a long time. With plants under short days (9 hr.), which were investigated in blue, yellow, and red light, no flowering occurred, but the petioles were considerably elongated in the blue compartment. This vegetative effect is especially remarkable because it shows that the shortening tendency of the blue and violet light, and also the elongating effects of yellow and red observed in most plants, do not show up in this rosette plant; rather, the reverse is true. Flowering of plants in continuous red light was greatly speeded up by intercalation of exposures to blue light for 9 hr. once a day or once in two or three days.

When 10 hr. exposure daily in white light is extended by 4 hr. or 8 hr. of either red or near infrared, or by 4 hr. of each in succession, the plants receiving infrared show elongation and subsequent flowering, those with red alone do so at a much slower rate, if at all. The succession of 4 hr. in both spectral regions causes elongation and flowering, irrespective of the sequence of the two periods. According to the authors, the flower initiation occcurring in the long run in high-intensity red light as exclusive irradiation may be attributable to a small admixture of near infrared radiation (37, p. 394); this explanation is deemed unlikely for the flowering in the violet and blue compartments since "near infrared is completely absent in violet and the contamination in blue is smaller than in red" (37, p. 394).

It had been found by Åberg (13) that the spectral dependence of morphogenetic light reactions depends on the light intensity. He stated that red light was most effective in inhibiting the most extreme elongation, whereas for further inhibition of elongation at higher intensities, the violet-blue region of the spectrum was more effective. Our knowledge about this high intensity reaction is rather limited, mainly because of experimental difficulties in obtaining high intensities of monochromatic light over large areas and for long periods. The available results are in general agreement with those of Wassink, Stolwijk & co-workers discussed above (29, 31, 32, 33, 37). Kleshnin (38) studied the effect of "monochromatic" light on growth of different

plant species. He used unfiltered light from single-phosphor fluorescent lamps and observed the highest dry weight in red light, when the spectral regions were given at intensities of from 25,000 to 40,000 ergs/cm.²/sec. In all but a few cases he found a reduced apparent efficiency of photosynthesis in green and blue light, in experiments lasting from 17 to 64 days. In experiments of such duration it is quite possible that indirect (developmental) effects distort the photosynthetic efficiency. Moshkov (39) isolated three spectral regions: violet-blue and yellow-green from a mercury lamp, and red from incandescent lamps. Using *Perilla* and *Citrus*, he observed only a very small gain in dry weight in red light, as compared with that in green and blue. This is probably associated with the large amount of near infrared present in his red light. A similar reaction was observed in tomato by Stolwijk (31) when red light with a large amount of infrared was given. The addition of infrared seems to upset the balance between leaf growth and stem growth at the expense of future photosynthetic capacity.

In other experiments, with *Perilla*, Moshkov (40) has observed an effect of long wave length infrared radiation. When the short day plant *Perilla* was given a 14 hr. day in white (incandescent) light, with a 10 hr. night at a temperature of 20–22°C., flower buds were observed after 17 days. When the night temperature was raised to 30–35°C., no flower buds were formed until after 37 days of treatment. If the plants were given 20,000 to 40,000 ergs/cm.²/sec. of infrared between 1 and 2.5μ during the dark period, at a temperature of 30°C., flower bud formation did not occur up to the end of the experiment after 60 days of treatment. To our knowledge this is the first time that a specific effect of long wave length infrared has been reported in full-grown plants. Unfortunately, Moshkov does not give any spectral characteristics; the high energies he used make it possible that he had an appreciable amount of near infrared radiation so that it is difficult to evaluate the significance of this experiment.

Evidence for a Reversible Reaction Between Red and Infrared Absorbing Pigments Involved in Photomorphogenetic Reactions

As mentioned in the Introduction, the scope of this article does not allow a discussion in great detail of the spectral dependency of seed germination and of flower initiation. Some data, however, mainly obtained by Borthwick, Hendricks and co-workers should be mentioned since they gave rise to theoretical considerations which these authors find applicable to a very wide range of photomorphogenetic phenomena. This is clearly indicated in the following quotation: "Effect of light upon germination of some seed is one manifestation of a very general, possibly universal, phenomenon controlling living processes." This quotation alludes to the findings, originally made by Flint & McAlister (19) many years ago and verified by the above authors (41) that red light (maximum at 660 mμ) promotes germination of certain varieties of lettuce seeds whereas infrared light (maximum between 710 and 750 mμ) inhibits this germination. In the experiments by Borthwick

et al. the seeds to be inhibited are first irradiated with a wave length region between 580 and 660 mμ to allow 98 per cent germination. Thereafter they were irradiated in various wave length regions. Those to be promoted were irradiated directly after darkness. Germination was checked after two days at 20°C. following the irradiation. The action spectra for promotion and inhibition were determined [(41), Fig. 1]. Among the most important further observations were that after a certain time of imbibition of the seed, the sensitivity of promotion of germination decreased, while that of inhibition accordingly increased, and further, that only the last of a sequence of red and infrared radiations determines the germination response. The authors interpret the first observation as an indication of a relation between the photoreceptive pigments, and the latter as "striking evidence of the immediate formation of one pigment from the other" (41, p. 664). Thus, two pigments appear to be involved (with other reactants) in the action of light on germination. The change in response with duration of imbibition is evidence that a reactant other than the pigment is also involved. Some evidence was obtained from dark reactions achieving essentially the same shift in the involved pigment system as infrared radiation. The authors assume a relationship of the following nature between the two pigments involved:

$$\underset{\substack{\text{max. absorption at 650 m}\mu,\\ \text{dormant seeds}}}{\text{Pigment} + RX} \quad \underset{\text{infrared radiation, darkness}}{\overset{\text{red radiation}}{\rightleftharpoons}} \quad \underset{\substack{\text{max. absorption at 730 m}\mu,\\ \text{germinating seeds}}}{\text{Pigment-}X + R}$$

RX probably is an acceptor which, in its turn, may exchange groups with substrate molecules in dark reactions.

In another paper, Borthwick *et al.* (24) found this situation applicable to flower initiation in *Xanthium* under certain experimental conditions. In earlier work the maximum effectiveness in controlling flowering by interruption of the dark period was found to be in the region of 630 to 660 mμ, both for short or long day plants (42, 43, 44, 45). The authors state that thus far they had not recognized the region of 700–760 mμ as effective in control of flowering [(24), p. 930]; radiation at wave lengths greater than 720 mμ was found to be ineffective in suppression of floral initiation. The authors now established, however, that radiation in the region from 721.5 to 745 mμ was able to restore the tendency to floral initiation after this tendency had been destroyed by unfiltered irradiation from a filament lamp. Thus, here also, the antagonism between red and infrared radiation was observed [cf. also (23)], and the spectra, determined previously by the authors, are considered to represent "the integrated absorptions of the two compounds, Pigment and Pigment-X" of the above equation. Just as with seeds, variation in red sensitivity was found to be coupled inversely with variation in infrared sensitivity. Moreover, it is interesting that half an hour of red radiation at the end of the light period increases the required duration of the following dark period for floral initiation in *Xanthium*, whereas a similar infrared treatment has the reverse effect. These observations are explained on the basis of the

above equation, assuming that "Pigment" is associated with floral initiation, and "Pigment-X" with prevention thereof. (Normally, the dark period slowly provokes the reaction from right to left; the different radiation treatments provoke additional shifts in the directions indicated in the equation.) This is another illustration of the dependence of the critical daylength on spectral properties of the light (cf. Introduction).

Piringer *et al.* of the Beltsville group (46) find that inhibition of flowering by red and its promotion by near infrared[5] can be effected by less than 1 min. of each type of radiation. The intensity of light used is not indicated; it is mentioned, however, that the intensity determines the required duration. According to the authors, the action spectra in the red and near infrared regions are the same for light effects on several different phenomena: seed germination, photoperiodic phenomena, and also for yellow pigment formation in a tomato fruit (47). The latter pigment does not develop in darkness. A brief daily irradiation with light of very low energy at about 650 mμ during the 5 to 10 day period of ripening is sufficient for pigment formation. This effect can be removed by subsequent irradiation at about 730 mμ. The analysis given earlier for the short day plant *Xanthium* is reported to hold *mutatis mutandis* also for the long day plants *Hyoscyamus niger* and *Hordeum vulgare;* in these cases red radiation is promotive and infrared radiation inhibitory to flowering. The observation on *Hyoscyamus* is curious in view of the results reported by Stolwijk & Zeevaart (37) who grew these plants in coloured light cabinets (after short day pretreatment in white light, mostly natural daylight). They found rapid flowering in blue light (with a slight admixture, i.e., less than one per cent of near infrared and no red) and much slower flower initiation and flowering in the red cabinet unless a considerable quantity of near infrared was admixed (see above). Our present knowledge seems insufficient to present an analysis of the relation between the two results, obtained along very different lines of approach.

[5] These authors recently have substituted the indication "near infrared" by "far red." The present reviewers, however, are inclined to retain the original indication, considering 700 mμ to be the boundary between red and infrared, just as 400 mμ is the boundary between violet and ultraviolet [see also (65), discussed below]. The argument of visibility for the human eye extending beyond 700 mμ does not appear very convincing since its long wave length boundary is not clearly defined as it depends on the energies considered. Moreover, where does "far red" begin? Based on the response of the human eye, 680 mμ is certainly "far red" already. The wave length of 700 mμ would seem to represent a fairly acceptable separation between "red" and "infrared" with respect to the response of the human eye as well as in reference to photosynthesis and to the reactions discussed by the Beltsville group. The only sensible alternative would seem to be to extend "far red" from 700 mμ to 1 μ; "infrared" might then be defined as the region without any known specific photobiological and photochemical (?) effect. But this also seems arbitrary, unless it could be clearly stated why such effects are absent from the latter wave lengths. Moreover, such a distinction is not applicable to violet and ultraviolet, and would thus disturb the symmetry between ultraviolet and infrared.

According to Hendricks & Borthwick (48), the thermal reaction from right to left (in the above equation) in lettuce seed has a half time of about 24 hr. at 35°C. With near infrared radiation the reaction proceeds in the order of seconds and is temperature independent, as is also its reversal by red radiation. Promotion of germination by red light is reversible by near infrared within 4 hr., decreasingly so up to 12 hr. and no more thereafter. For floral initiation in *Xanthium* the time interval for reversal of inhibition caused by red radiation is less than 30 min. A remarkable observation is that near infrared radiation given for 1 min. after red radiation is able to reverse the effect of the latter, but fails to do so when continued for 30 min. To the reviewers this observation seems unexplainable from the above equation without further specified assumptions. It may well serve as a bridge toward understanding reactions upon long time irradiations in restricted spectral regions as observed by Stolwijk & Zeevaart (37). It seems that, especially in this field, much more factual knowledge must be collected before a full explanation is possible (see also pages 381 and 394–95.

In addition to the above form of the equation, Borthwick *et al.* (49) have discussed another possible form, viz., $P \rightleftarrows P^*$. In the more recent paper by Toole *et al.* (50) these investigators come back to the earlier form. They suppose that the substances RX and R are closely associated with the pigment (in order to account for the absence of a temperature coefficient), and that the photosensitivity in both directions is determined by the amounts of RX and R[6] (Assuming such a close connection between these substances and the pigment *sensu stricto*, it is difficult to see any real difference between the assumed version and $P \rightleftarrows P^*$ until the concentrations of the reactants, including the pigments can actually be determined.) Borthwick *et al.* (49) speculate on the energy level diagram of the postulated reactions. It is assumed that the reversible change ($P \rightleftarrows P^*$) goes through a common triplet state. Earlier (44), the pigment had already been supposed to be of the phycocyanin type, on the basis of the action spectrum of the "red reaction."

Effects of Light Quality on Growth of Etiolated Plant Material

An advantage of the use of etiolated material in spectral studies is that only low light intensities are required, often over only short periods, which makes it possible to improve the spectral purity, and to use simpler equipment for the irradiations. The sensitivity of etiolated material can be illustrated by the fact, that both leaf expansion in etiolated peas (51) and mesocotyl inhibition in *Avena* (52, 53) give measurable responses to irradiation with a total energy in the order of 1 to 10 ergs/cm.^{2} in the region of maximum efficiency.

[6] Recently, the Beltsville authors (50) have substituted AR for RX and A for R, which, for the sake of simplicity, has not been introduced into our text. So, for example in (50, Fig. 4), the notations would be R and RX according to our text.

Except for phototropism and related phenomena, which have a maximum response in the violet-blue spectral region, other light-influenced growth reactions in etiolated plant material show the greatest response in the red part of the spectrum, e.g., leaf growth and internode inhibition in etiolated peas (51), and in bean seedlings (17), internode inhibition in etiolated normal and albino barley seedlings (45), mesocotyl inhibition in *Avena* seedlings (52, 53), and the opening of the plumular hook in bean seedlings (54). In all these reactions red light was more, and sometimes much more, effective than blue light. For some of the reactions accurate action spectra have been measured and although there are some discrepancies and uncertainties, the available action spectra indicate that these reactions all may have essentially the same photo-receptive mechanism.

Borthwick *et al.* (45) measured the action spectra for stem growth inhibition in dark-grown seedlings of barley. They used both potentially green and albino barley seedlings and found essentially the same action spectrum for both materials. It may be remarked that in these experiments they were unable to get a red-reaction in the region around 480 mμ, and also that the red-reaction boundary was displaced towards slightly longer wave lengths. It is remarkable that they were able to get inhibition up to 740 mμ, a wave length already beyond the point where the reversal reaction should be most effective (735 mμ). This would seem to indicate that in this material no complete reversal is possible. If one wishes to retain the scheme developed in (41) it would mean that the curve of *RX* at all wave lengths would be below the curve of *R*.

Recently, Withrow and co-workers measured a detailed action spectrum for the straightening of the plumular hook in etiolated bean seedlings (54). Their action spectrum for the red reaction is essentially the same as the spectra published by Borthwick *et al.*, but it shows more details, especially in the region between 400 and 550 mμ. Withrow *et al.* find the red maximum around 660 mμ, with very small maxima at about 500, 435 and 400 mμ. In the action spectrum for the infrared reversal they found two maxima, at about 710 and 745 mμ, with a slight minimum around 725 mμ.

Goodwin & Owens (52) working with mesocotyl inhibition in *Avena* seedlings, found an action spectrum for 10 per cent inhibition which was very similar to the action spectra obtained by Borthwick *et al.* When 30 per cent inhibition was used as a reference, they found a completely different action spectrum with a sharp peak at 589 mμ, a very low response in the red beyond 620 mμ, and even lower activity at wave lengths less than 580 mμ. Although it is very probable that the mechanism responsible for 10 per cent inhibition is identical with that described by Borthwick *et al.* for a number of reactions, it is unknown whether the inhibition is reversed by infrared radiation. It seems that for 30 per cent inhibition the cooperation of another photoreceptor system is required which shows a peculiar absorption curve. Goodwin & Owens suggest that the mechanism characterized by this action

spectrum operates on cell elongation, whereas the 10 per cent inhibition system operates via inhibition of cell division.

Liverman & Bonner (55) have demonstrated that growth in length of *Avena* coleoptile sections which is promoted by red light is reversed by subsequent infrared irradiation. This infrared reversal only obtains if indoleacetic acid had been added to the system. Infrared radiation *per se* could not reverse the growth promotion by red light. The auxin could be added either before or after the exposure to red light. Double inverse plotting of their results indicated that the infrared acts like a competitive inhibitor, and that red light increases the growth rate without affecting the reaction constant of the primary reaction in which the auxin is involved. These findings led Liverman & Bonner to suggest a hypothesis involving a cyclic process in which auxin would combine with a receptor. The receptor would be formed from an auxin-nonreceptive precursor under the influence of red light. The auxin-receptor complex is formed independent of any light, as soon as the auxin and receptive entity are brought together. The complex would be responsible for the various plant reactions. This active complex is degraded slowly in the dark, and very rapidly by infrared, to auxin and, as has been suggested, an auxin-nonreceptive precursor, thus completing the cycle.

This attractive hypothesis unfortunately loses plausibility because of inconsistencies between the presented data and the Lineweaver-Burk treatment. Moreover, the reaction seems to have required very high light energies. Although Liverman & Bonner did not indicate the intensities they used, it can be inferred from description of the light sources that the total light energies must have been several powers of ten higher than normally required in this type of work.

Liverman & Starr (56) are reported to have found that leaf expansion in bean seedlings shows a reaction similar to the one described for *Avena* coleoptile sections.

Thus, in the last few years, it has become increasingly evident that a large number of hitherto unrelated reactions of etiolated plant material to light have the same photo-receptive mechanism and possibly the first few "dark-chemical" steps in common. An analysis of the interaction of the red-infrared light system and auxin would probably increase our understanding not only about the mechanism of the red-infrared system, but also of the action of growth substances in general.

A reaction that stays somewhat apart from those discussed in this section is the effect of various spectral regions on the development of potato tuber sprouts studied by Wassink *et al.* (57). In the dark these sprouts show an extreme elongation. In the light their elongation is depressed, especially in red and near infrared; in red they contain appreciable quantities of chlorophyll, whereas in near infrared they do not. In the published experiments they also showed strong inhibition in blue and violet, and somewhat less inhibition in yellow and green. The latter feature thus far has not been fully

corroborated in subsequent unpublished experiments so that this point still requires further investigation.

Effects of Light Quality on Photosynthesis in Higher Plants

In studies of morphogenetic effects of light quality in green plants, photosynthesis takes part in the over-all growth process. Both in fundamental studies and in comparative studies of the efficiency of different light sources in artificial lighting, it is important to know to what extent light quality has an indirect effect on growth via photosynthesis. Hoover's measurements (58) indicated that blue and red light were more efficient than light in the yellow-green region. When the Hoover curve is replotted to indicate relative quantum efficiency [Burns (59, 60)] it is found that blue light is even slightly more effective than red. Relative efficiency in the green around 550 mμ was about 50 per cent of that in the blue-violet around 450 mμ under Hoover's experimental conditions. Gabrielsen (61), in *Sinapis alba* found a reduction in photosynthetic efficiency both in blue and green, as compared to red. In his case the relative reduction in the green was smaller than in Hoover's experiments. In growth experiments with tomatoes Stolwijk (31) found that the dry weight formed over long periods in different spectral regions of equal intensity was related directly to the number of quanta. This indicates that under his experimental conditions, green and yellow light were just as effective as red and blue in photosynthesis. The spectral data on absorption, transmission and reflection of light by different green leaves, as collected by Rabideau, French & Holt (36) are illustrative of the way in which such different results can be obtained. It is evident that, depending on anatomical structure, pigment concentration, leaf thickness, number of layers of leaves on a plant, irradiation intensity, and other factors, a variety of relations between wave length and photosynthetic efficiency can be expected to obtain. Table I gives some values for leaves of three different species, for a single and a double layer of leaves. The relative photosynthesis is given, assuming that every quantum absorbed is effective. Evidently such an assumption is not generally justified, especially for example when large amounts of anthocyanins are present [Gabrielsen (61)]. From a comparison of the "quantized" Hoover curve (59, 60) with the absorption data obtained by Rabideau *et al.* (36), one may get the impression that absorption by carotenoids does not decrease the quantum efficiency to any large extent.

It is clear that the amount of depression of photosynthetic efficiency in the green region can vary appreciably depending on the species and the experimental conditions. In general the depression is relatively small, smaller than would be expected from Hoover's data, for example, although the effect of two or more layers of leaves as compared with a single layer is smaller than might be expected. As can be seen from Table I, a major factor in determining this is the differential reflection of the leaf in different wave length regions.

When the effect of different spectral regions is studied over long periods,

TABLE I

ABSORPTION, REFLECTION AND TRANSMISSION OF DIFFERENT LEAVES, BASED ON DATA FROM RABIDEAU, FRENCH & HOLT (36). THE RELATIVE PHOTOSYNTHESIS HAS BEEN COMPUTED UNDER THE ASSUMPTION THAT EVERY QUANTUM ABSORBED IS EQUALLY EFFECTIVE IN PHOTOSYNTHESIS, AND THAT PHOTOSYNTHESIS IS NOT LIGHT-SATURATED, AN ASSUMPTION WHICH IS NOT ALWAYS JUSTIFIED

Species	Wave length in mμ	One layer of leaves				Two layers of leaves			
		Reflection	Transmission	Absorption	Relative Photosynthesis	Reflection	Transmission	Absorption	Relative Photosynthesis
Lactuca sativa	450	0.17	0.01	0.82	1.03	0.17	0.00	0.83	1.01
	550	0.35	0.25	0.40	0.50	0.38	0.06	0.56	0.68
	670	0.18	0.02	0.80	1.00	0.18	0.00	0.82	1.00
Brassica	450	0.14	0.06	0.80	0.99	0.14	0.00	0.86	0.99
	550	0.30	0.20	0.50	0.62	0.33	0.04	0.63	0.72
	670	0.13	0.06	0.81	1.00	0.13	0.00	0.87	1.00
Ficus	450	0.06	0.01	0.93	0.99	0.06	0.00	0.94	1.00
	550	0.12	0.03	0.85	0.90	0.12	0.00	0.88	0.94
	670	0.06	0.00	0.94	1.00	0.06	0.00	0.94	1.00

other effects may obscure the spectral dependence of photosynthetic efficiency, e.g., changes in leaf shape, leaf area, or the ratio of leaf area to nonphotosynthetic tissue. Moshkov (39) reports reduced dry weight of plants grown in red light as compared with those grown in equi-energetic blue or green light. This is almost certainly due to a large amount of near infrared radiation contaminating his red light. His red light was obtained from incandescent lamps with a red glass filter. A similar effect of admixture of much infrared was noticed by Stolwijk (31) in experiments with tomatoes.

A very interesting observation has been made by Voskresenskaya (62). This author studied the products of photosynthesis, using C^{14} as a tracer. Comparison of the relative amounts of the different products formed in photosynthesis in blue and red light respectively, showed that in red light relatively smaller amounts of amino-acids were formed. Especially alanine was formed preferentially in blue light. For a phenomenon such as this, photo-reception must take place separate from the primary light reaction in photosynthesis. It is conceivable that this phenomenon is related to the plasma growth substances reported by Ruge (63), in which case it might be visualized that a regulatory mechanism could channel the products of photosynthesis as early as 90 seconds after primary CO_2 reduction.

PLANT IRRADIATION

A few words should be said concerning research in the borderline field between plant physiology and horticulture that is mostly referred to as "plant irradiation." The literature in this field is very extensive and cannot

be discussed here. Roodenburg (64) collected 80 titles excluding photoperiodism, in 1954 and 1955 only.

Economic viewpoints play a considerable role. The principal question is: How can seedling growth, vegetative development, flowering, and fruiting of flower and vegetable crops be promoted at the lowest cost, yielding plants of the best appearance at the (economically) most suitable moment, either in combination with the natural greenhouse illumination or purely by artificial light? The connection with the present review is that in various cases the suitability of a light source for main or additional irradiation depends on its formative effects under the conditions given. Since in most cases the spectral range emitted by the light sources used is wide, it is not always easy to discover the scientific background of the observed effects. Often rather unclear and, at first sight, contradictory results are reported which can only be understood by a much more fundamental attack on the problem. Especially work on problems such as, for example, whether 40 or 60 watt incandescent lamps should be used, at what distance the light source should be placed from the plant, whether 8 or 12 hr. light should be given a day, whether high pressure mercury vapour tubes are preferable over fluorescent tubes, and similar questions, has often yielded rather unclear results. In general, one may say that photosynthesis as such is likely to depend mainly on the total energy given between 400 and 700 mμ. The spectral characteristics of the main or supplementary light exert formative effects and may influence leaf development which may in turn express itself in the photosynthetic yield.

The Dutch Committee on plant irradiation has advised (65) characterizing light sources by their emission in various bands which have been chosen according to spectral sensitivities in plants, so that a more meaningful comparison of various light sources is possible. The wave length bands are: greater than 1μ, 1μ to 0.7μ, 0.7μ to 0.61μ, 0.61μ to 0.51μ, 0.51μ to 0.4μ, and less than 0.4μ. The 0.7μ boundary was originally 0.72μ, which was changed at an international meeting during the Fourteenth International Horticultural Congress, The Hague, 1955. The change was especially made in view of the recent discovery by Withrow *et al.* (54) that the formative, infrared reaction (cf. above) has maxima at about 0.71 and 0.73μ. In a second paper (66) the Dutch Committee has indicated how the required data can be collected and has advised expressing them in absolute (energy) units.

A few papers in the plant irradiation field will be briefly mentioned. Ruge (67) reports that, depending on the duration of the experiments, two different types of fluorescent lamps proved to be most satisfactory. The "warm-white" type emitting more red, was most satisfactory in short-term experiments whereas the "day-light" type, with more blue—and consequently less quanta—proved to be most satisfactory in long-term experiments. Relative changes in the concentration of the different pigments could also be responsible for such long term effects, although Stolwijk (31) did not find a change in either the relative or the absolute amounts of leaf pig-

ments in tomato plants which had grown over a long period in blue or red light.

Bouillenne & Fouarge (68) were able to specify the mixtures of phosphors in fluorescent tubes and arrive at an optimal spectral energy distribution for growth of tomatoes and lettuce in their "Phytor" lamp. It is interesting to compare photographs of tomato plants grown in light of unfiltered monophosphor fluorescent tubes, i.e., "blue," "green," and "red" tubes [Van der Veen (69)], with photographs of plants obtained in corresponding, narrow spectral regions published by Wassink & Stolwijk (32). The latter demonstrate much more clearly the formative effect of the absence of short wave length radiation. The sensitivity of various strains still remains to be investigated. In unpublished work of our laboratory, Bensink has found that the magnitude of stem elongation of lettuce seedlings on day-length extensions with blue or near infrared radiation [cf. (23)] depends very much upon the strain used.

Borthwick & Parker (70) using beets and *Hyoscyamus*, found incandescent light much more photoperiodically active than "daylight" or white fluorescent light. This is readily explainable by the higher red content of the incandescent light.

Reinders-Gouwentak *et al.* (71) grew tomato plants under a 7½ to 9 hr. day in light of high pressure mercury vapour tubes (Philips HO 2000). Upon extension of such a light period by some hours of weak incandescent light, the plants reacted "unfavourably," producing a more spindly growth. The authors concluded that, for normal development, tomato requires a long period of uninterrupted darkness, and further—from comparison of 6, 7½ and 9 hr. illumination periods—that "the minimum period necessary for good growth is already longer than the maximum period of light of high intensity that may be lengthened with light of low intensity without injury to the tomato." It would seem that in these statements, the importance of the spectral composition of the light used for day-length extension has been underestimated. The technique they used will work like a day-length extension with low intensity of near infrared, a condition in which Wassink *et al.*, in various cases, found conspicuous internode elongation (20, 23, 32). Had the authors used supplementary light predominantly composed of any other sort of radiation (except, perhaps, pure blue) their findings might have been quite different.

Usually flower initiation (72) started later in series of plants that had received a supplementary low-intensity light period except, for example, when a brief basic light period was followed by only a brief extension. Day-length extension by weak incandescent light and by red light filtered from fluorescent light yielded similar results.

Roodenburg (73) speaks of a "physiological day-length" which, under natural conditions, differs for various plants owing to their different photoperiodic sensitivity with regard to spectrum and intensity. His general statement that incandescent light has "a much more daylengthening effect

than daylight itself, owing to the excess of the short wave infrared rays of the tungsten filament lamp" appears somewhat dangerous. This may be true for certain elongating effects (cf. above), but it may well be that the red content is more important than the infrared content for lengthening days with respect to flower initiation in various plants, except, probably, crucifers (cf. the discussion on pages 376–85 inclusive). Moreover, it seems dangerous to list the formative effects apparent in plants in predominantly blue radiation as "short day effects" just because plants grown in short days under natural conditions show these effects.

The few papers discussed above may serve to indicate that reactions of plants to broad band irradiations are necessarily complicated. It is often difficult to see which is the predominant effect and to which wave length region, either in the main or supplementary irradiation, the effect is attributable. These behaviours, however, are important for horticultural purposes and, moreover, their analysis often opens physiologically interesting viewpoints.

Effects of Light Not Directly Measured as Growth

Some effects of light cannot be measured directly in terms of growth but express themselves in reactions which may well affect the ultimate growth rate. Analysis of such phenomena can give information about the steps between primary light absorption and ultimate growth response.

Montfort & Rosenstock (74), and Rosenstock (75) studied the effect of light of different spectral regions on respiratory rate in non-chlorophyllous tissues. They found a temporary increase in respiratory rate following ultraviolet, blue, and infrared irradiation. This increase was found in discs of potato and *Asparagus*, seeds of *Cucurbita*, bulbs of *Allium cepa*, and white leaves of *Acer negundo*. It can be concluded from their data that red, green and yellow light annulled the stimulation caused by infrared, blue and ultraviolet. The increase in respiration was linearly related to light intensity, and reached a maximum about 1 to 1½ hours after the start of the illumination. The respiratory rates expressed as per cent of the dark rates, reached a maximum of 140 in potato discs and 170 in discs from onion bulbs. White leaves of *Acer negundo*, which were light adapted by exposure to light before the experiment, did not show any appreciable increase in respiration upon irradiation with ultraviolet, blue or infrared. Adaptation to darkness took about seven days, after which these leaves showed the increase in respiration discussed above. The stimulation of respiration was temperature dependent; in potato discs the largest stimulation was found at 21°C; at 10°C. it was much smaller, and at 33°C. it was negligible. Rosenstock used two methods for obtaining coloured light: isolation of spectral regions by filters (at an intensity of 3.6×10^5 ergs/cm.²/sec.) and by the use of filters with successive short wave length cut-offs (at an intensity of 1.4×10^6 ergs/cm.²/sec.). It is evident that these intensities are extremely high.

Somewhat later, Leopold & Guernsey (76), who do not seem to have

been aware of Rosenstock's work, reported effects of red and infrared radiation on respiration of leaf sections, lettuce seed, *Avena* mesocotyls, and stem sections of etiolated peas. The respiratory rates were determined manometrically, and although Leopold & Guernsey do not state the light intensities used, they must have been considerably lower than those applied by Rosenstock. In Leopold & Guernsey's experiments the light intensities must have been well below the compensation point of respiration in green leaves (of the order of 10^3 to 10^4 ergs/cm.^{2}/sec.), as they did not find a reduced oxygen evolution in red light in barley leaves. Notwithstanding these differences in experimental material and techniques, the results obtained in the investigations of Leopold & Guernsey and of Rosenstock are remarkably similar, but an important discrepancy is found in the effect on seeds. From Meischke's data (77) it can be inferred that germination in *Cucurbita* seeds is promoted by red light, and very probably inhibited by infrared. These seeds are very similar in this respect to the lettuce seed used by Leopold & Guernsey for which Borthwick *et al.* established the same characteristics. However, Rosenstock found increases in respiratory rate in *Cucurbita* seeds as a results of infrared radiation, while Leopold & Guernsey found an increase of respiratory rate in lettuce seed as a result of red light, an increase which was reversed by infrared applied subsequently.

A study of light-induced changes in the viscosity of protoplasm was made by Virgin (78, 79). This author measured an action spectrum of these light-induced changes, using the displacement of cell contents in *Helodea* leaves in a centrifugal field. The spectrum shows a wide, possibly double peak in the region between 400 and 500 mμ, with maxima at about 450 and 475 mμ. In a later study, Virgin (79) gives a slightly modified curve, with a broad maximum between 460 and 490 mμ. Both at 400 and at 500 mμ the effectiveness is less than one-fifth that of the maximum. These results are in good agreement with those obtained by Bottelier (80) who studied protoplasmic streaming in *Avena* coleoptiles. If a relationship between protoplasmic viscosity and the reaction causing internode inhibition in light-grown plants in light of high intensity (32) can be established, the mechanism studied by Virgin may be of considerable value in further studies of the light growth reaction because of its speed and relative simplicity.

Additional Remarks on Photoreceptive Systems and the Mechanism of Photomorphogenetic Action of Light on Plant Growth

Action spectra and the more qualitative studies on the effect of light quality are of importance for giving information about the primary light absorbing pigment. These studies also indicate whether two phenomena are related, in which case the experimentally simpler reaction can be chosen for further work.

An important difficulty arising in the interpretation of action spectra is the possibility of other pigments having a screening action, and also the possibility of a changed quantum efficiency at various wave lengths. Ap-

parently, various primary photoreceptors occur at very low concentrations (27, 45) and, especially in green plants, one may expect a considerable distortion of the action spectra, as compared with the true absorption spectrum of the pigment. No definite differences in the action spectra for green and chlorophyll-free material so far are known which enable a quantitative judgement of this problem.

Reviewing the field of phototropism, Galston (81) could not arrive at a final conclusion regarding the nature of the pigment responsible for primary light absorption, notwithstanding the availability of very accurate action spectra. If the blue-sensitive light reactions are based on a photodynamic destruction of substances, *in vitro* studies can hardly be expected to yield convincing evidence in this question, as these studies usually imply a degree of homogenization which departs far from the situation obtaining *in vivo*. Under such conditions many pigments normally not associated with the physiological response may show photodynamic activity. Galston & Baker (82) have described experiments with stem sections of etiolated peas, in which they studied the effect of riboflavin added to a system of stem sections in indoleacetic acid solution, in light and in darkness. They find that the stimulation of growth by the auxin in the solution is almost completely annulled by adding riboflavin in the light. The action spectrum for this reaction followed very closely the absorption spectrum of riboflavin. The action spectrum for photo-inactivation of indoleacetic acid by a tissue-brei of stem sections was also very similar. However, Galston & Baker also measured an action spectrum for phototropic curvature of *Avena* seedlings which had resemblance to the absorption spectrum of β-carotene. If phototropic curvature is assumed to result from differential photo-inactivation of auxin, this would indicate that in the tissue-brei from pea stems, a pigment is active different from that connected with phototropic curvature of *Avena in vivo*.

In light reactions in light-grown plants, the region between 400 and 500 mμ is most effective in internode inhibition. From the results reported by Wassink & Stolwijk (27) and Stolwijk (31) it would seem that the most effective region is nearer to 400 than to 460 mμ as, in their experiments, internode inhibition is invariably stronger around 400 mμ than it is around 460 mμ. This does not point to a close relation between these reactions and phototropism but the data are not yet sufficiently detailed to arrive at a definite conclusion in this respect.

The reaction in *Hyoscyamus* as reported by Stolwijk & Zeevaart (37) may be related to the growth inhibition by blue light. This reaction also shows a region of maximum effectiveness nearer to 400 mμ than to 460 mμ. The similarity of the effects of violet-blue and those of near infrared in this reaction is very intriguing. It seems that, under the conditions of these experiments, the reaction occurring under the influence of blue and infrared is not reversed by red light. This follows from some of the experiments made by Stolwijk & Zeevaart, in which it was evident that even when a large excess of red light was supplied over long periods following an exposure to

XUM

blue light, or simultaneously with a low intensity of continuous infrared irradiation, the blue-infrared reaction still proceeded without any reversal. It is not yet possible to decide whether the blue and the infrared effect arise from the same pigment system.

The relation of these effects to the red-infrared reversible reactions studied by Borthwick *et al.* does not yet seem quite clear. In the long day plant *Hyoscyamus*, these authors (44) find a promotive effect on flowering produced by red radiation, while under the conditions of Stolwijk & Zeevaart's experiments with plants exposed exclusively to narrow spectral region radiations, blue-violet and near infrared have a definite promotive effect which is lacking, or at least very much slower, in red. Piringer *et al.* (46) studied the reversal of the photoperiodic response in *Hyoscyamus* by near infrared and found that the red reaction was reversible by near infrared, provided the infrared radiation was not given over too long a time. If more than the required amount of infrared was given, the reaction was not reversed. Our knowledge at present does not yet seem to be sufficient for comparing results obtained by highly different techniques. In various cases of internode elongation after supplementary irradiation, Wassink and co-workers (20, 23, 27 to 33) found blue light to be active in the same direction as infrared, in a number of responses. Later experiments [Stolwijk (30, 31)] indicated that part of that response was due to a very small near infrared contamination. After complete removal of the near infrared from the blue light however, a significant elongation of the internodes of *Cosmos* still was found. The older work by Flint & McAlister (19) on seed germination also indicated that blue light has the same kind of effect as infrared. They used a monochromator for isolation of spectral bands, so that their results are not likely to have been affected by infrared contamination in the blue. Differences in technique may account for discrepancies in the results.

Attempts at isolation of the pigments so far have been unsuccessful. Borthwick *et al.* (45) in etiolated barley seedlings did not find any specific absorption around 640 mμ. Stolwijk (27) measured absorption spectra of extracts of leaves of etiolated pea seedlings without finding any specific absorption in the near infrared, although the plants showed a definite response to this radiation. Todd & Galston (83) isolated a pigment from *Poa* seeds which showed high absorption peaks at 410 and 667 mμ. The fluorescence maximum of this pigment *in vivo* was found to be at 665 mμ. These authors obtained indications that the pigment, after purification, was very similar to, or identical with methyl pyrophaeophorbide *a*. Todd & Galston believe they have indications that this pigment is associated with the red-infrared response of seed germination because the amount of extractable pigment is lower after irradiation of the seeds with red light, and because the light sensitivity in different varieties of lettuce seed was correlated with the amount of extractable pigment. However, Todd & Galston did not find a corresponding increase in an infrared absorbing form of the pigment after irradiation of the seeds with red light; in fact, they did not find any near

infrared absorption at all. They did find a fluorescence peak at 716 mμ, indicating that the pigment has an energy level in the near infrared, but this was only found in the purified pigment. Their pigment also shows a very strong absorption in the blue-violet, which seems hard to reconcile with the present knowledge of the action spectrum for light effects on seed germination. Drumm (84) extracted a pigment very similar to that found by Todd & Galston from chlorophyll-deficient *Tradescantia*. Efforts to extract this pigment from green sprouts of the same species were unsuccessful, so that Drumm assumed that methyl pyrophaeophorbide is a product which accumulates only when chlorophyll formation is absent because of functional abnormalities, or when growth occurs in darkness.

Blaauw-Jansen (85) studied a pigment (or pigment mixture) obtained from cell material of *Chlorella* cultures. It is claimed to be a chlorophyllide derivative and inhibits the growth of certain bacteria in the light (or gives rise to the formation of a growth inhibitor in the medium in light). For no very good reason, the substance is tentatively called "chlorellin." There seems to be no definite reason for the author's tentatively linking up this substance with the phenomena under discussion in this review. There are no data on the exact nature of the substance or on its absorption spectrum (except that "orange light," "Schott filter OG2," with light source not indicated, was more destructive than "green light"). This red sensitivity obviously is the main reason for the mentioned speculation. But it is most likely that Blaauw-Jansen's substance will have a blue or violet absorption maximum higher than the red one, as the detailed studies on absorption spectra of chlorophyll and porphyrin derivatives by Stern *et al.* indicate [cf., e.g., (86) p. 589]. On the other hand, the higher plant reactions referred to have a blue sensitivity lower than the red sensitivity, and where a considerable blue sensitivity has been found, it is connected with a near infrared sensitivity rather than with a red sensitivity. There is, of course, no reason for doubting that a similar substance might as well be isolated from chloroplasts of higher plants. The described effect seems to be an interesting case of the not too well explored field of "photodynamic actions" in microorganisms. From the data presented, however, it is a big jump to connect it with formative light effects in higher plants.

The extensive studies by Borthwick *et al.* on the nature of the red-infrared reversible light reaction have been discussed above. Borthwick *et al.* (49) attribute the greatest probability to the first of two possible reactions:

$$\text{I}\quad \underset{(660\ m\mu)}{\text{Pigment } P} \rightleftarrows \underset{(735\ m\mu)}{\text{Pigment (isomeric) } P^*}$$

$$\text{II}\quad \text{Pigment} + \text{reactant} \rightleftarrows \text{Changed pigment} + \text{changed reactant}$$

Their main reason for doing this is the absence of any temperature dependence in their multiple reversal experiments. They compare reaction II to the change from protochlorophyll to chlorophyll which, however, is not known to be reversible. Judging from their published data, however, the exclusion of reaction II might not yet be fully justified. Virgin (87) has

XUM

demonstrated that the temperature dependence of the conversion of protochlorophyll to chlorophyll—which has been shown to be a bimolecular reaction—does not become evident with dark periods of the order of 10 milliseconds, i.e. much shorter than the irradiation-darkness schedule used by Borthwick *et al.* In a later paper (50) Toole *et al.* use the second alternative to explain further results in seed germination. The pigment in the infrared absorbing form is considered to be the biologically effective form. This is concluded from the fact that inhibition of the flowering response in *Xanthium* by red light can only be reversed by infrared if the infrared is given within 30 min. after the red irradiance, indicating that the infrared absorbing form is withdrawn from the photochemical reaction by a dark reaction with about that time constant.

Conclusion

This review has tried to cover some of the main literature since the previous review in this series (3). The main increase of knowledge appears to have been along the following lines: (*a*) The discovery of photoperiodic and formative effects of near infrared radiation in full-grown green plants (20, 23) and the indication of antagonism between the action of different spectral regions (20, 23). (*b*) The recognition that various, at first sight not closely related, effects show the same spectral sensitivity (24, 46), suggesting close similarity or identity of the underlying pigment system. (*c*) The demonstration that this pigment system shows a reversibility between a red absorbing and a near infrared absorbing form (24, 49, 50), the final form of which determines the ultimate physiological behaviour.

Some major points that have been attacked but require further study and are likely to yield interesting results in the near future would seem to be: (*a*) The effects of the spectral composition of the "main light period," and its interaction with day-length extension, especially with respect to the short wave length regions (e.g., 32). (*b*) The relation between the formative effects of spectral regions and auxin metabolism. (*c*) The identification of the underlying pigment system (S).

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XUM

RECENT ADVANCES IN PLANT PHYSIOLOGY IN THE U.S.S.R.[1]

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Investigations in plant physiology have received extensive attention in the Soviet Union. Interest in our country in this branch of botany began in the second half of the 19th century with the work of the Russian scientists Famintsin (1), who developed the chemical trend in plant physiology, and Timiriazev (2), who is famous for his investigations of chlorophyll and photosynthesis. The latter scientist was a talented popularizer and did much to evoke interest in plant physiology; he also stated the main problems of this science. Later on, plant physiology was further developed by Palladin (3), with his work on the enzymatic nature of respiration, Kostychev (4), well known for his work on respiration, fermentation, and photosynthesis, Liubimenko (5), who did much in investigating chlorophyll and other plant pigments, Kholodnyi (6), investigator of plant growth and development, and Maximov (7), well known for his work on cold resistance and water relations in plants. Maximov was the founder of the ecological school in plant physiology in our country. Finally, one should mention, among many, Prianishnikov (8), the author of classical investigations on nitrogen metabolism of plants, and Sabinin (9), the originator of a new direction in the investigation of the physiology of roots.

The development of plant physiology in the U.S.S.R. is closely related to the general development of this science throughout the world. However, plant physiology in the Soviet Union possesses some distinct features of its own; in particular, perhaps more than in any other country, it is considered not only as the science concerned with the life of plants but also as the theoretical basis of agriculture. Physiological investigations in the U.S.S.R., therefore, are usually carried out on cultivated plants and are frequently connected with the solution of practical agricultural problems. As a result, a great deal of attention is paid, in such works, to the influence of the environment on plants, to the resistance of plants to unfavorable factors, to root nutrition as the most convenient means of influencing the crop yield, to the postharvest period which is connected with ripening and storage, to regulation of growth and development, and to similar problems. A characteristic feature of the work of Soviet physiologists is the study of the complex relations between the various parts of the plants on the basis of research on component physiological processes. General problems of plant physiology,

[1] The survey of the literature pertaining to this review was concluded in October, 1955.

in particular, those dealing with photosynthesis, translocation, secondary transformations, and plant development also evoke great interest.

Among the larger institutions in the U.S.S.R. in which investigations in plant physiology are pursued one might mention the Timiriazev Institute of Plant Physiology, Academy of Science, Moscow, which, besides carrying out research work, also coordinates work in plant physiology carried out in the U.S.S.R. by organizing annual discussions and conferences; the Institute of Agrochemistry and Plant Physiology, Academy of Science, Ukr. S.S.R. in Kiev; Biology Institute Academy of Science, Byel. S.S.R. in Minsk; Agricultural Institute Academy of Science, Uzb. S.S.R., Tashkent; Institute of Botany Academy of Science, Georg. S.S.R. in Tbilisi; Institute of Botany Academy of Science, Arm. S.S.R.; Erevan; Institute of Breeding in Leningrad; Biology Institute in Yakutsk; Departments of Plant Physiology in the State Universities in Moscow, Leningrad, Kazan, Petrozavodsk, Kharkov, Sverdlovsk, Saratov, Gorky, Irkutsk, and other cities.

The results of plant physiological research carried out in the Soviet Union during the last few years are presented in the present paper. To give a more complete description of the work the author has attempted to cover various aspects of plant physiology using mainly the literature which has appeared during the last four to five years. Because of space limitations, a complete review cannot be given here; therefore only those investigations which are characteristic of established trends of research in plant physiology in the Soviet Union or in which clear-cut and important results have been obtained will be discussed.

Because of space limitations the results of foreign authors are not mentioned although many of them are obviously related to the problems discussed in this review and are cited in the Russian literature.

Photosynthesis

Photosynthesis studies carried out in the U.S.S.R. cover the following topics: photochemistry of photosynthesis, structure and composition of chloroplasts, products of photosynthesis, and the role of photosynthesis as a factor determining crop yield.

Terenin (10, 11, 12) suggested that, on absorbing light, the plant pigments undergo a transition to a long-lived biradical state. Krasnovskii *et al.* (13 to 16) proved that chlorophyll solutions can undergo reversible photoreduction and demonstrated the "accumulation" of the quantum energy in active photo-products of chlorophyll.

By applying spectral and electrometric methods Evstigneiev & Gavrilova (17) were able to divide the photoreduction of chlorophyll into two elementary stages. According to Krasnovskii the reversible photoreduction of chlorophyll observed in these investigations underlies the sensitized transfer of hydrogen to various acceptors and in particular to the dehydrogenase prosthetic groups, riboflavin and pyridine nucleotides (18 to 21). The possible participation of dehydrogenases in photosynthetic reactions was demonstrated by using the method of competitive inhibition (22). A Ukrainian

XUM

group of investigators (Dain and co-workers) detected the fact that Fe-derivatives of chlorophyll are capable of undergoing reversible photochemical reduction (23, 24, 25). Related to these experiments are those of Boichenko (26, 27), who demonstrated the positive role of iron-containing compounds in the photoreduction of CO_2 by isolated chloroplasts. Vinogradov & Teiss (28, 29) also carried out important work in photosynthesis; by using the isotope O^{18} they demonstrated that water is the precursor of the oxygen evolved during photosynthesis.

In 1923, Liubimenko (30) assumed the existence of chlorophyll protein complexes in chloroplasts. Godnev & Osipova (31, 32) working along this line showed that the affinity between chlorophyll and protein may be quite variable. It was also found that the state of chlorophyll in the plastid grana changes during greening of the plant (33, 34). Godnev and colleagues found that chlorophyll formation in dicotyledons can proceed in the dark. The relation between chlorophyll synthesis and the tricarboxylic acid cycle was studied by these authors with the aid of $C^{14}O_2$ (35, 36). Chloroplast enzymes were studied in detail by Sisakyan and co-workers (37). The work of Tabentsky is devoted to the study of the dependence of the structure of chloroplasts on their age and the conditions of nutrition (38). The influence of illumination conditions and nitrogen nutrition of the plants on the chemical composition of chloroplasts was demonstrated by Osipova (39). The phototactic movement of tobacco leaf chloroplasts was studied by Babushkin (40) in relation to various parts of the spectrum.

Nichiporovich and colleagues (41, 42) based their investigations on the assumption that, at some early stages preceding the formation of carbohydrates, the photosynthetic products diverge in different directions and form various products. By using C^{14}-tagged carbon dioxide and sugar and ammonium sulphate containing N^{15}, Andreeva (43) obtained evidence in favor of direct photosynthetic formation of proteins in chloroplasts. She also showed that alanine, serine, and glycine are amino acids formed in the highest concentrations in leaves of *Nicotiana rustica* during photosynthesis. It has been shown that the composition of the products of photosynthesis depends on the species of the plant (44, 45) and on the spectral quality of the light. In particular, predominance of red light leads to a relatively more intensive formation of carbohydrates while the short-wave blue light favors the formation of amino acids and proteins [Voskresenskaia (46)]. Finally, as Zalenskii (47) has shown, the temperature also affects the composition of photosynthetic products. All these facts seem to indicate that the diversity of the direct products of photosynthesis is the source of the biochemical specificity of plants.

Nichiporovich (48, 49, 50) investigated the conditions determining the activity of the photosynthetic apparatus in plants growing under natural conditions. He established the relation between the number of leaves and their photosynthetic activity. The data thus obtained can be used to determine the optimal distribution of agricultural plants. Several authors studied the dependence of photosynthetic productivity of agricultural plants on

watering conditions, temperature, and fertilization (51, 52, and others).

Baslavskaya (53) studied the photosynthesis of aquatic plants and demonstrated that the productivity of this process can be raised by introducing mineral fertilizers into the water. The work of Zalenskii (47) is devoted to the investigation of photosynthesis in plants under natural conditions and, in particular, at the high altitudes in the Pamir mountains (at 3860 m. above sea level).

Kursanov, Kuzin & Mamul (54) showed, with the help of C^{14}, that CO_2 and carbonates absorbed from the soil by roots reach the green tissues and, in the presence of light, are transformed into sugar and proteins. Kuprevich (55) had previously found that carbon dioxide, entering the plant through excised stems, together with water, can be used by plants in photosynthesis. Further investigations showed that root absorption of CO_2 is connected with β-carboxylation of keto acids and with the movement of dicarboxylic acids towards the leaves (56, 57). This process is closely related to the absorption of ammonium salts from the soil and to the synthesis of amino acids in roots (58).

Field experiments showed that, if carbonic acid is introduced in the soil in the form of ammonium or potassium carbonate, the crop increases by 10 to 15 per cent; sugar beet in the Latvian S.S.R. (59) and other plants (60) were investigated. The positive influence of carbonic acid absorbed by plants from the soil was also detected by Samokhvalov (61) who carried out laboratory and field experiments in the Ukraine.

A large amount of attention has been directed to the problem of determining the physiological basis of cultivating plants under artificial light; this problem is important for growing vegetables and decorative plants in winter. The results of these investigations have been presented in a number of articles (62 to 65) and in the books of Kleshnin (66), Krasinskii (67), and Moshkov (68).

The Physiology of Root Nutrition

Investigations have shown that the uptake of nutritional elements by roots depends on whether the elements are in an adsorbed-bound state or in solution as ions of salts (69 to 72). This difference is not restricted to the adsorption process but also depends on the chemical mechanism of the plant. It has been shown by Ratner & Akimochkina (73, 74) that if beets, wheat, etc. are fed with potassium in the adsorbed-bound state the amount of organic acids in the leaves increases by two to four times compared with the amount of acids in plants which received potassium in chloride or sulphate compounds. Vladimirov (76), developing Prianishnikov's (75) idea of the influence of the state of soil nutrients on plant metabolism, showed that plants rich in organic acids mainly use the ammonium ion from NH_4NO_3, while plants rich in carbohydrates intensively assimilate the nitrate ion. This author also found that ammonium nitrogen favors the formation of reduced products in the plants, such as rubber and ethereal oils, whereas nitrates intensify the synthesis of acids. By varying the conditions of mineral

nutrition of cucumbers, melons, and maize, Minina (77) could considerably change the ratio between the number of male and female flowers in plants.

The investigations of Chailakhian (78) into the influence of nitrogen fertilizers on the development of long- and short-day plants were continued by Ratner (79). The latter author showed that in long-day plants very small doses of nitrogen fertilizers stimulate flower initiation; higher doses retard reproductive development. In short-day plants much higher nitrogen doses are required for inhibition of reproductive development.

Sabinin (80) demonstrated in a number of papers the important role of roots as organs in which many vital compounds are synthesized. Klechkovskii & Bagaiev (81) found that nucleoprotein synthesis in plants is possible only if inorganic phosphorus is assimilated through the roots. It was shown with the aid of isotopes that plant roots participate in the synthesis of nucleoproteins, lipoids, and proteins (82, 83, 84). The work of Shmuk and colleagues (85), who had previously demonstrated the role of tobacco roots in nicotine synthesis, was confirmed by Il'in (86), who used C^{14} for this purpose. Kursanov, Tuieva & Vereshchagin (87) and Kolosov & Ukhina (88) detected that roots are capable of synthesizing various amino acids. These results agree with those of Tumanov & Vinokur (89), who showed that roots, as metabolic organs, require high temperatures, and also with the experiments of Vinokur (90), who established that, even if the roots do not fulfill any absorptive function, they are nevertheless necessary for the growth of lemon shoots. Kursanov (91), who studied the biochemical activity of the air roots of *Ficus* arrived at a similar conclusion. The significance of roots as organs participating in the turnover and transformation of matter in plants has been considered in a survey paper (58). Thus, during the last few years much attention has been directed toward roots as organs which biochemically participate in the general metabolism of plants.

Considered from this point of view, supplementary mineral nutrition of plants by foliar application cannot be considered as equivalent to nutrition through roots. The results of a study of phosphate nutrition of plants carried out by Tuieva & Samoilova (92, 93) confirm this conclusion. Nevertheless, several authors have obtained positive results with foliar nutrition. By powdering sugar beets with potassium and phosphorus salts several days before harvest, Iakushkin & Edelstein (94) attained better movement of the sugar from the leaves to the roots as shown by the higher sugar content in the beets. According to Uchevatkin & Borodulina (95), (Uzbek S.S.R.) phosphorus nutrition by foliar application favorably affects the opening of cotton bolls and the formation of cotton fibers. Petinov & Pavlov (96) found that the protein content of wheat grain can be raised by foliar nitrogen nutrition. Dadykin (97), working in Northern Siberia, showed that in the vicinity of eternally cold territory, foliar nitrogen nutrition of cabbage can be effective.

The possibility of direct utilization of organic substances from the soil has also been discussed in the literature. Application of isotopic, chromatographic, and sterile culture techniques has permitted a more accurate estima-

tion of the relation between autotrophic and heterotrophic types of nutrition in plants to be carried out. Krasilinikov (98) found antibiotics to be absorbed by roots. By analyzing the sap of bleeding plants Ratner (99) demonstrated the rapid absorption and translocation of a number of vitamins of the "B" group to the aerial plant organs. Penetration of amino acids into the plant by the same pathway was demonstrated by Shavlovskii (100). Using tracer carbon, Merenova & Kuzin (101) observed the absorption by roots of organic substances from decaying plant remains. Some of the organic compounds exert a stimulating (102) or formative (88) effect on the plants but independently they cannot serve as a source of nutrition as only small amounts are absorbed.

Kolosov & Ukhina (88) found that the synthesis of alanine, glutamic acid, and serine in plants can be increased if the roots are treated with small quantities of the products of the metabolism of soil microorganisms. About ten enzymes capable of affecting the organic complex of the soil were found in root excretions [Kuprevich (103, 104)]. Ratner & Samoilova (105) demonstrated under sterile conditions that the phosphorus of glycerophosphate and other organic substances as a rule can penetrate the plant only after it is split off by enzymes located on the root surface. This group of investigations elucidates the nature of the interaction between plants, microorganisms and organic substances of the soil.

Parallel with the investigations of the physiology of the root system research has been carried out on the utilization of fertilizers. These investigations will not be considered in the present article. Some of the results are presented in Sokolov's book (106). Investigations on the application of microelements were also carried out. Shkol'nik (107) and Katalymov (108) studied the influence of boron on plants; Vlasiuk (109, 110) investigated the influence of manganese on the metabolism and crop yield of plants in the Ukraine; and Peive (111) determined the distribution of cobalt and copper in the soil of Latvia and the requirement of various regions for microfertilizers. Godnev & Terentiev (112) studied the efficiency of microdoses of copper in the turf soils in Byelorussia, and Kedrov-Zikhman (113) the influence of cobalt and molybdenum in the same district.

Translocation of Organic Substances

Kursanov & Zaprometov (114) found that the uptake of amino acids by maturing wheat and rye seeds exceeds the uptake of water by 50 to 100 times. The same authors showed that, with respect to conduction of amino acids, living stem tissues possess "polarity" which is attributable to the different absorbing properties of cells located at opposite ends of the stem segment (115). It had previously been shown that absorption of organic substances by plant tissues is accompanied by an enhancement of respiration (116). These data lead to the conclusion that translocation of organic substances in plants is not identical with the passive movement of a solution but is based on the active transfer of molecules as a result of a specific type of physiological activity of the conductive tissues (117). Further investigations

XUM

of translocation of organic substances were carried out from this point of view.

Kursanov & Turkina (118) found that respiration is as high as 5000 $\mu l.O_2$ per hr. per gram fresh weight in the fibro-vascular bundles of the phloem of sugar beet, *Plantago*, and other plants. When sucrose or other organic substances pass through the conductive tissues, the increase in respiration is even higher. Turkina & Dubinina (119) showed that the specificity of respiration of the fibro-vascular bundles is attributable to the presence of a very active cytochrome oxidase system, which is activated by the moving sugars. If a section of the conduction path is inhibited by CO, it loses its property of conducting organic substances (120). This prompts one to seek an intimate relation between translocation of substances and the functioning of the cytochrome oxidase system. It has been shown that the movement of substances in plants is not accompanied by noticeable changes of their concentration or composition in the conduction paths. The moving components can, therefore, be detected and studied only by combining the isotopic and chromatographic methods (121). It was shown in this way that the products of photosynthesis move from the leaves along the conductive tissues with a velocity between 30 and 100 cm. per hr (121, 122, 123). Further, in *Cucurbita pepo* the larger part of the assimilates initially move downwards towards the roots irrespective of the position of the leaf on the plant (120) and the products of photosynthesis undergo further secondary transformations in the roots (87, 88). The predominant role of roots has also been emphasized in the papers of other authors (85, 89, 90, 124).

Kursanov & Vyskrebentseva (123) showed that a mixture of sugars, organic acids, and phosphoric esters move towards the seeds along the conductive tissues of cotton. Sucrose and hexosephosphates quantitatively predominate in this mixture. This fact is the basis of the hypothesis that sugars move in the form of their phosphoric esters (125). A mixture of sugar and amino acids moves from the leaves of pumpkin to the fruits, the relative amounts of these two components depending on the age of the fruit (120). The main component moving from the leaf to root in sugar beet is sucrose [Okanenko (126)]. According to Turkina (127) this disaccharide is the first free sugar formed in the leaves of sugar beet during photosynthesis. It penetrates the conductive bundles with greater ease than monosaccharides and moves to the roots with greater speed. Pavlinova (128) showed that, in the fibro-vascular bundles of sugar beet, simple sugars undergo isomerization and transformation into sucrose. The role of uridine-diphosphate-glucose in the synthesis could not be demonstrated. Phosphatase, starch phosphorylase, and a weakly active invertase have also been detected in the fibro-vascular bundles of sugar beet. It has been suggested that, in the sugar beet plant, sucrose is synthesized in the leaves and conductive bundles. The root is not capable of synthesizing sucrose from simple sugars (129). Direct experiments carried out with tagged sugars confirm this opinion (130, 131). This point has been the subject of a discussion (132).

Pontovich (133) used C^{14} to study the turnover of substances in imma-

ture poppy capsules. The first stage of the process is the evolution of CO_2 by the respiring seeds into the internal cavity of the fruit. The CO_2 then diffuses to the green wall, undergoes photoreduction, and, as a product of photosynthesis, returns through the placenta to the seeds. This turnover promotes the removal of CO_2, which hampers synthesis of fats. It leads to a high concentration of oxygen in the inner cavity and decreases the amount of organic substances expended in respiration. Simple field methods in which radioactive carbon was employed permitted the movement of metabolites to be studied in wheat raised in the irrigated lands of the Volga region [Zholkevich (134)] and in potatoes raised in the Far North [Zhurbitskii (135)].

Respiration

Zalenskii (47) made a parallel study of photosynthesis and respiration at various temperatures and arrived at the conclusion that these two processes are closely related in the plant. A study of oxidative enzymes and respiration (mostly in vegetables and fruits) was carried out by Rubin and co-workers in connection with problems of ripening and storage. On the basis of their investigations these authors (136, 137, 138) concluded that various oxidase groups are important for plants as far as resistance of plants to fungal and bacterial diseases is concerned (139). In another series of investigations Rubin and co-workers studied the dependence of respiration and various oxidative systems in plants on temperature (140 to 143). These investigations led Artsikhovskaia & Rubin (144) to the conclusion that plant respiration is highly adapted to natural temperature variations; in the opinion of the authors, this adaptation arose during the phylogenesis of the plant.

Respiration of fibro-vascular bundles was studied by Kursanov, Turkina & Dubinina (118, 119, 145) in connection with the translocation of substances in the plants. Kolesnikov continued his investigations on the role of C_2-compounds in plant respiration. He demonstrated that glyoxylic acid participates in the reduction of nitrates (146) and also that in leaf extracts, oxidation of glyoxal can take place with subsequent formation of glyoxylic acid (147). This author also observed the formation of glycine from glyoxylic acid (148). Thus, according to Kolesnikov, glyoxylic acid is the center of many transformations.

Continuing their previous investigations on the respiration of grain (cereals) in connection with the problem of storage, Kretovich & Prokhorova (149) established two respiration periods of which the first corresponds to postharvest ripening of the grain and is accompanied by noticeable losses of storage substances. The influence of various factors on the intensity of respiration of stored grain is considered in a different paper by the same authors (150).

Enzymatic Syntheses in Plants

Investigations of the action of enzymes in living plants carried out in the U.S.S.R. between 1936 and 1941 by the vacuum infiltration method contrib-

uted toward the establishment of a close connection between enzymology and plant physiology. The results of these investigations have been generalized in a number of survey papers (151, 152, 153). Oparin's concept (154) that adsorption on cell structures is important for synthetic reactions in the plant was further developed in the work of Kursanov (155, 156), Oparin & Kaden (157), Sisakian *et al.* (158, 159, 160) and others, who pointed out that the adsorption of enzymes by tissues is connected with the nature of the processes taking place in the cell. A critical analysis of these investigations is presented in a recent review (161).

Sisakian & co-workers studied the enzymatic activity of plastids extracted from plants (37, 162). These authors were able to prove the presence of a large number of enzymes and to study their variation with time in the plastids (163, 164, 165). It was also shown that part of the enzymes in plastids are in an inactive state but become active upon disintegration of the protein stroma of the plastids (166, 167). The synthetic function of isolated chloroplasts was also demonstrated (168).

Investigations on biosynthesis in plants are carried out in the U.S.S.R. mostly on living tissues by introducing initial compounds or the suspected intermediary products. Among other methods vacuum infiltration is widely used for these purposes. Kursanov & Pavlinova (169) demonstrated that in sugar beet leaves the synthesis of sucrose from maltose proceeds at a higher rate than from a mixture of glucose and fructose. The same authors found that synthesis of sucrose in plants is accompanied by enhancement of respiration; thus in wheat sprouts twice as much oxygen is used in the synthesis of a milligram of sucrose from glucose and fructose than from a mixture of maltose and fructose. This seems to indicate that glucose residues activated by 1:4,α-glycosidic bonds can participate in the synthesis of sucrose by enzymatic transference (170). In this connection, in analogy with hexosophosphoric esters, di- and polysaccharides are considered to be compounds containing activated sugar resiaues which are utilized in transference reactions (141). Lisitsin (171), who studied the transformation of glycosides in lilac and ash, arrived at the conclusion that these compounds play an important role in the activation of sugars, in particular, in oxidative transformations.

Kotelinikova investigated the synthesis of starch and detected hexokinase (172, 173) and apyrase (174) in potato tubers. However, no phosphoglucomutase could be detected. It was shown that, along with tuber growth and increase of starch content, the activity of starch phosphorylase also increases. The synthesis and decomposition of starch in potato tubers at various stages of growth was investigated by Rosenfeld & Shubina (175) and Petrova *et al.* (176). Stepanenko *et al.* (177) have reviewed the work on starch synthesis in potatoes.

An investigation of glucofructosan in various organs of Jerusalem artichoke was carried out by Strepkov (178). This author found that fructosans moving from the leaves to the storage organs undergo polymerization, which also continues in the tubers.

A detailed study was made on cotton fibers at various development

periods (179). It was established that cellulose synthesis in cotton fiber takes place on the twenty-fifth day after fertilization of the flower, about 1 mg. per hr. of cellulose being formed per gram dry weight of fiber (180). During the period of wall thickening, the velocity of cellulose synthesis is limited by the supply of sugars. Cellulose synthesis can be accelerated and continued if a glucose solution is introduced into the boll. It was demonstrated that mainly sugars and phosphoric esters enter the growing fibers from the leaves and green walls of the bolls (123). It has also been shown that the molecular weight of cellulose increases as the fibers mature (181).

Paleiev (182) found that during the maturing of the seeds of rye and other cereals, the mechanical tissues of the stems undergo partial delignification; their cellulose is converted into sugar (up to 30 per cent) and is used as a nutrient for the maturing seeds. This phenomenon is related to the "lodging" of corn which is observed on certain soils when the yield is high (183). Enzymatic splitting of cellulose in a higher plant was demonstrated in *Elodea* (184).

As early as the 1930's, S. Ivanov and N. Ivanov showed that the fat composition of plants depends on the climate and on cultivation conditions. New evidence confirming and extending this concept has been obtained in the last few years. This topic has been reviewed recently in Sharapov's book (185). Many authors have found that considerable doses of nitrogen fertilizers adversely affect fat synthesis, whereas phosphorus and potassium fertilizers give a positive effect. The literature on this problem has been reviewed by Iermakov (186) and very recently by Prokof'ev (187).

Cytophysiological investigation of seeds of flax, sunflower, mustard, and other oil plants showed that the transformation of starch into fat takes place in the plastids themselves and is accompanied by the formation of a colorless zone of intermediary products in the starch grain [Sveshnikova (188)]. According to Zhdanova & Ivanova (189), the period of transformation of starch into fat coincides with a sharp rise in the activity of aldolase and this, in the opinion of the authors, indicates the glycolytic nature of the initial transformations. Later on, lipase becomes preponderant.

The work of Soldatenkov *et al.* (190) is devoted to the investigation of organic acids in germinating seeds of cereals. These authors found that during germination the acid content increases three to five times, mostly as a result of di- and tricarboxylic acids. A number of authors have pointed out that there is a direct dependence between the uptake of cations and accumulation of organic acids in tobacco (74), sugar beet (191), and other plants. New data confirming the functioning of the Krebs cycle in the potato plant have been obtained by Prokoshev & Minina (192, 193). Organic acids in the conductive tissues and fibers of cotton plant were investigated in connection with the problem of synthesis of cellulose in this plant (103). Malonic acid has been detected in the tubers of Jerusalem artichoke (194). This problem requires further study.

The discovery of transamination by Braunstein & Kritzman (195, 196) and others led to a more detailed study of the biosynthesis of amino acids in

plants. Kretovich & Bundel (197) demonstrated the synthesis of alanine from pyruvic acid in shoots of lupine, pumpkin, and corn; this takes place either by direct amination of the acid or by transamination in the presence of glutamate or, less readily, in the presence of aspartic acid (198). The same authors showed that in sprouts the synthesis of aspartic acid from oxalacetic or fumaric acids proceeds in the presence of ammonia (199, 200). Finally, glutamic acid can be synthesized from α-ketoglutaric acid and ammonia; cozymase activates the process (201). In brief, both living sprouts and purified preparations showed that, along with reamination, direct amination is also important for the synthesis of amino acids. On the other hand, Olenicheva (202) found in several plants an enzyme system which carries out associated reamination and deamination in the presence of phosphopyridoxal. Kolesnikov (203) found that in plant leaves glyoxylic acid can participate in reamination of glutamic and aspartic acids.

Aspartic and glutamic acids are oxidized in plant tissues directly (204, 205) as well as in a secondary way by means of the polyphenol-quinone system which is catalyzed by polyphenoloxidase (206). Attachment of an amide group to dicarboxylic amino acids renders the latter less susceptible to oxidation (205). Glutamine introduced into nitrogen-deficient wheat seedlings is utilized more rapidly in protein synthesis than asparagine (207). This fact was interepreted as signifying the incorrectness of the existing opinion that both compounds are physiologically equivalent. Related to this problem is the investigation of Kretovich, Bundel & Uspenskaia (208) on the dicarboxylic amino acid content in germinating seeds of wheat, rye, and beans.

Kretovich *et al.* (209, 210) have shown that, when the storage protein albumin is extracted from pea seeds by lyophilyzing, this protein, which may be electrophoretically divided into four components, displays enzymatic activity, acting as amylase, phosphatase, and dehydrogenase, and reductive aminase of keto acids.

Primary synthesis of amino acids has been detected in plant roots absorbing ammonium fertilizers (87, 88); over 12 amino acids have been identified. Part of these move with the sap from the roots towards the upper organs and is utilized in protein synthesis. The ability to synthesize many amino acids has also been detected in the air roots of *Ficus sp.* (91).

A dependence between nucleic acid synthesis in plants and the plant development was found by Konarev (211). By combining nitrogen and phosphorous nutrition of the plants it was possible to influence considerably the formation of nucleic acids. Petinov & Pavlov (96) showed that if, during the milk stage of seeds, the plants are sprayed with a 3 per cent solution of ammonium sulphate, protein synthesis in wheat seeds could be enhanced. This method is being tested in South East regions of the U.S.S.R. in artificially irrigated regions.

Much attention is being directed to the investigation of the biosynthesis of substances of secondary origin. The formation of phenol rings was studied in tea leaves (212). Kriukova showed that the latter contain about 1 per

cent inositol in the free and bound state (213). All of the inositol is in the *meso*-form (214, 215). It was shown by vacuum infiltration that tea leaves can synthesize *m*-inositol from glucose and even more rapidly from sucrose and several glucosides (216). Maltose, ramnose, pyruvate, glycerine aldehyde, and glycolic aldehyde are not suitable for this synthesis.

Introduction of *m*-inositol, quinic acid, or quercite into leaves leads to the formation in the cells of polyphenols with the oxy-group in the 1, 3, 5, or 1, 2, 3 positions (216). These data confirm the hypothesis of biosynthesis of the phenol ring from activated glucose through *m*-inositol (212). Zaprometov (217) showed that simple catechins (1-epicatechin and *d,l*-catechin) are the first to be synthesized in germinating tea seeds. The formation of galloylcatechins is a secondary process. Biosynthesis of catechins in the tea plant has also been studied in connection with irrigation [Lebedev (218)] and with the age of the leaves [Jemukhadze (219)].

Manskaia & Bardinskaia (220) studied the formation of lignin in aspen and pine. They found that oxidative polymerization of coniferyl alcohol takes place under the influence of oxidases. Using Bardinskaia's method of separating lignin components (221), Manskaia & Bardinskaia detected aromatic aldehydes peculiar to higher plant lignin in the cellular walls of *Sphagnum* which does not form true lignin (222).

Continuing his investigations (85) which indicated the participation of roots in the synthesis of nicotine in tobacco, Il'in (86) found, with the help of $C^{14}O_2$, that carbon dioxide absorbed by roots from the soil is utilized in the synthesis of nicotine. It was also demonstrated that in excised tobacco shoots nicotine, nornicotine, and anabasine synthesis ceases but recontinues when new roots are formed. Mashkovtsev *et al.* (223) discovered the ability of tobacco root tissues to destroy nicotine. These investigations confirm the viewpoint that alkaloids participate in the metabolism plants. A study of the alkaloids of *Senecio platiphyllus* led Areshkina (224) to the conclusion that alkaloids exist in plants in two forms (oxidized and reduced) which are significant in the plant redox processes.

The composition and distribution of glycoalkaloids in various sorts of potatoes and tomatoes were studied by Prokoshev and colleagues (225, 226, 227). In contrast to alkaloids of the nicotine and tropane group, solanine, demissine, and tomatine apparently are formed in leaves (228). Petrochenko (229) discovered in potato tubers a specific enzyme which can decompose solanine.

Investigation of the biosynthesis of rubber was studied, as previously, in kok-sagyz, and several other representatives of the Compositae. Prokof'ev (230, 231) and Zhdanova (232) found that the synthesis of rubber takes place in the latex vessels with the aid of special plastids. The molecular weight of rubber in the assimilating parenchyma is 10 to 20 thousand and, in the lactiferous ducts, 100 to 200 thousand [Prokof'ev (233)]. Belikov (234), Mashtokov (235), and others found that rubber accumulates with age, but is not consumed in the plant. If kok-sagyz roots are dug out and stored the

amount of rubber may increase on the average by 20 per cent as a result of its synthesis [Neiman & Dobrovolskaia (236); Sobolevskaia (237)].

Later on, Neiman, Prokof'ev & Shantarovich (238), using C^{14}-labeled sugars, demonstrated that carbohydrates are utilized in the synthesis of rubber. In several investigations by some Ukrainian workers an attempt was made to determine the optimal conditions for accumulation of rubber in kok-sagyz. The necessity of a long day, moderate temperature, and correct combination of fertilizers (nitrate at the beginning, and ammonium at the end, of vegetation) were especially emphasized (239, 240, 241).

L'vov (242) investigated the conditions favorable for accumulation of ethereal oils in mint (*Mentha piperita*) and some other plants and arrived at the conclusion that these reduced products are formed as a result of oxygen deficiency which compels the plant to utilize the oxygen of its organic compounds.

The dependence of the vitamin content of plants on geographical conditions and type of plant was studied in the U.S.S.R. mostly before 1945. These investigations laid the basis of a new trend in plant selection (increase of the vitamin content of plants). As a result, new races of plants were selected; wild rose which contains 27 to 30 per cent ascorbic acid in the fruits (243), black currant containing up to 400 mg. per cent ascorbic acid (244), gladiolus containing in the leaves up to 1700 mg. per cent ascorbic acid, pumpkins containing up to 37 mg. per cent carotene in the flesh of the fruit (245), etc. Catechins, which constitute 15 to 25 per cent of the tea leaf, were found to possess a very distinct vitamin P action (246, 247). This new material is used in industry to obtain medicinal preparations (248). Catechin obtained by Durmishidze & Bukin (249) from grape seeds also possesses vitamin P action.

In the work of a number of authors it has been shown that the biosynthesis of vitamins can be increased by wounding the plants; thus the amount of ascorbic acid [Prokoshev (250)], folic acid [Andreeva (251)], and riboflavin [Povolotskaia (252)] was increased in this way. These processes are paralleled by an increase in respiration and synthesis of proteins on the surface of the cut. Thiamine is not synthesized in injured plants (252).

Povolotskaia (253) showed that some riboflavin in the plant is firmly bound to the proteins and cannot be determined analytically without preliminary liberation. This compels one to revise the usual conception regarding the nutritive source of vitamin B_2. According to Povolotskaia & Bukin (254) this new form of riboflavin is a dinucleotide as well as the prosthetic group of succinic dehydrogenase.

Several investigators have discovered vitamin deficiency in plants. Rakitin & Ovcharov (255) found that pretreatment of cotton seeds with nicotinic acid yields favorable results. Similar results on beans were obtained in Lithuania by Dagis (256), who also found that thiamine raised the crop yield. According to Povolotskaia *et al.* (257) pretreatment of the seeds of wheat, rye, and peas with thiamine, riboflavin, and nicotinic acid enhances

germination and growth. In some cases nicotinic acid promotes the formation of leaves of fruit trees (258) and also the restoration of injured tissues (259). These investigations open new possibilities for investigation of the physiological role of vitamins in plants.

Resistance of Plants to Unfavorable Conditions

After the appearance in 1926 of Maksimov's well-known monograph (260) "The Physiological Basis of Drought Resistance of Plants," interest in this problem greatly increased in the U.S.S.R. As a result, several monographs have appeared which treat the problem of water regime from various viewpoints (153, 261 to 269, etc.). Most of the authors incline towards the view that drought leads to dehydration and overheating of the plant. Drought resistance itself is considered as an adaptive reaction of the plant which arises during ontogeny. Its extension and mode of appearance is determined by the evolutionary history of the given species or variety.

The investigations on the shift of enzymatic processes in withering plants carried out by Sisakyan and his colleagues (1936 to 1940) using the vacuum-infiltration method have been summarized in Sisakyan's book (153). At a later period Sisakyan & Kobiakova (270), working with wheats of varied drought resistance and also with sugar beet and pea, showed that when the tissues attain a certain degree of dehydration, the protoplasmic structures lose their ability to bind the enzymes (invertase and others) and as a result the enzymes go into solution and stimulate intensive hydrolytic decomposition. This phenomenon is observed in drought resistant plants only under conditions of high water loss.

During the last few years dependence of plant drought resistance on the colloid-chemical composition of the protoplasm and the related distribution of water in the cells has been studied extensively. Maksimov and his coworkers (271) demonstrated that after withering, the permeability and viscosity of the plant protoplasm remains low over a prolonged time.

Alekseev & Gusev (272 to 274) and Shardakov (275) examined the water regime of plant tissues from a thermodynamic viewpoint and suggested that the physiological activity of water is attributable to its various modes of interaction with the cell colloids. Moreover, as the properties of the protoplasmic colloids depend on nutrition and metabolic specificity, these authors attempted to influence the water regime of the plant by changing its biochemical activity. Alekseev & Gusev (273, 274) showed that the degree of hydration of the colloids of wheat leaves depends on the hydrophily of the absorbed cations. The influence of anions on the amount of colloid-bound water is more complex and depends on the age of the plant. The authors hope to raise the resistance of the protoplasmic colloids to dehydration by controlling the mineral nutrition of the plants.

According to Henkel & Margolina (276, 277) the capacity of plants to endure overheating is related to the high viscosity of the protoplasm and to the considerable amount of osmotic and colloid-bound water. Vasil'eva (278) found that when the temperature is raised to 34 to 39° C., in many

plants the distribution of the water changes, this being connected with an increase in the osmotic pressure of the cell sap and with a decrease of the water concentration of the colloids. One may compare these results with those of Alekseev (274), who showed that transpiration of wheat leaves depends directly on the amount of free water in the cells and is inversely proportional to the amount of bound water and the osmotic pressure. Maximov (279), who studied the swelling heat of wheat seeds found that if the heating is not uniform the water distribution changes and this leads to a redistribution of nutrients in the endosperm and embryo tissues.

The problem of early diagnosis of the water requirements of agricultural plants has attracted the attention of many plant physiologists. The importance of this problem is due to the fact that withering of the plant is a late symptom of profound alteration of metabolism, leading to unsatisfactory development and lower crop yields. Investigation of the period preceding withering has made it possible to determine optimal irrigation conditions and their dependence on soil and climatic conditions. The results have been presented in recent surveys by Ryzhov & Eremenko (280, 281), Petinov (282), Shardakov (275), Kruzhilin (283), and others. For early diagnosis of the water requirements of plants, various physiological properties were used (cellular suction pressure, concentration of cellular sap, osmotic pressure, and degree of stomatal opening); these properties reliably characterize the beginning of water deficiency in plants and can easily be determined by field methods especially developed for these purposes [Shardakov (275); Maximov & Petinov (284); Petinov (282); Lobov (285)]. Thus, it is possible to irrigate in accordance with the water requirements of the plants without causing them to wither and without making the expenditure of water too high. In practice, physiological methods of diagnosing water requirements of plants have been most extensively applied to cotton (275 to 286), vegetables (285), and wheat (282, 287).

In connection with the solution of this fundamental problem many special investigations were also carried out. Among these one should mention the work of Petinov (288, 291) on the amount of hydrophilic colloids (which ensure a more stable level of water content and of intensity of photosynthesis during the day). These increase in wheat and other plants grown in artificially irrigated land under conditions of optimal water supply and satisfactory mineral nutrition. Moreover, in such plants the translocation of assimilates from leaves to seeds increases (134, 289, 291). The additional development of phloem in the conductive bundles also favors the increased translocation (273). Zholkevich (290) found that in water-deficient wheat, respiration is enhanced but synthetic processes are inhibited. The author suggests that normal transfer of respiratory energy to energy-rich compounds is prevented in this case. As a confirmation of this viewpoint the higher activity of apyrase in withering plants is cited. Petinov (291) notes that physiological and anatomical variations appearing in wheat during ontogeny, as a result of the influence of irrigation, are enhanced in the second and third generations if the plant is cultivated under the same conditions. This leads to a

gradual increase of the productivity of wheat cultivated on irrigated land if seeds obtained from irrigated fields are successively sown.

Henkel (263) suggested a presowing method of increasing the drought resistance of such plants as wheat, sunflower, and maize which is based on Michurin's concept (292) of the plasticity of young organisms. This technique consists in the temporary (about two days) soaking of the seeds (up to 40 to 60 per cent of water relative to the air dry weight) and subsequent drying. Plants grown from such seeds give good crops under drought conditions. They are distinguished by higher viscosity and elasticity of the protoplasm and also by their more intensive photosynthesis and respiration (293).

The problem of resistance of plants to increased salinity continued to attract the attention of many investigators. Henkel & Shakhov (294) distinguish three types of plants according to the specificity of their mode of adaptation to saline soils: salt-accumulating plants (euhalophytes), salt-discharging plants (crinohalophytes), and salt-nonpenetrable plants (glycohalophytes). Burygin (295) moreover, distinguishes plants which concentrate salts. Several examples were cited to demonstrate that the metabolism of true halophytes and cultivated plants changes considerably under conditions of saline soils. This is usually expressed by a decrease in photosynthesis and respiration, inhibition of a number of enzymatic reactions, and variation of the water regime of the tissues, in particular, an increase of hydration of the protoplasmic colloids (295, 297 to 300). A colloidal-biological theory of salinity resistance of plants is given in Shakhov's book (296). According to Strogonov (301), high salinity leads to disintegration of the plant proteins and to accumulation of ammonia which adds to the toxic action of the salts.

Ratner (302) and Oknina (297) have emphasized that the uptake of highly concentrated salt solutions by plants is determined to a large extent by the intensity of the transpiration stream. The penetration of excessive salt restricts the uptake of necessary nutrients by the plant (303). In the opinion of Strogonov (301) and Henkel (304) the important adaptation reaction of plants to chloride salinity consists in attachment of the chloride ions to the proteins. Ratner & Akimochkina (73,74) emphasized the role of organic acids in binding excessive plant cations. Strogonov *et al.* (305, 306) demonstrated that the physiological activity of plants depends on the type of salinity. In particular, the predominance of sulphates in cotton plants favors the appearance of xeromorphism, whereas the accumulation of chlorides causes changes in the direction of halosucculence.

Shakhov (296) made some observations regarding the influence of salinity on the sex of plants. According to this author, staminate specimens of aspen, willow, and some other dioecious woody plants can grow on soils of higher salinity than pistillate specimens. Correspondingly, in monoecious plants, such as birch, salts lead to an increase of staminate and a decrease of pistillate flowers.

Many authors have concluded that repeated growing of plants on saline soils raises their resistance to salinity (296, 298, 300, 307, 308, 309). A result of this was the recommendation to sow in saline soils seeds obtained from

plants raised under similar conditions. Exploiting the high plasticity of the embryo meristematic tissues Henkel (299) proposed a method of increasing the salinity resistance of cotton and several other plants by pretreating the seeds for a short time with a solution of NaCl. This simple technique was checked under field conditions and was found to be effective for soils containing high concentrations of chloride salts (310).

Tumanov (311) has reviewed the work on frost resistance carried out in the U.S.S.R. up to 1951; this survey supplements an earlier monograph by the same author (312).

In one of his last papers Maksimov (313) gave a new critical analysis of the theory of plant freezing and proposed the concept of protective substances. According to this author, the direct cause of the death of plants at low temperatures below 0°C. is connected with the formation of ice which injures the protoplasm. Samygin (314, 315) suggested that the formation of small ice crystals can cause imperceptible damage to protoplasm, which is amplified upon thawing and leads to death of the cells. According to this author, lethal effects in onions attributable to low temperature take place only if the ice crystals are formed within the protoplasm itself. Henkel & Margolina (316), on the other hand, discovered that the tonoplasts of cucumber leaves can be injured at low positive temperatures (above 0°C.) which excludes the possibility that ice is the cause of the damage. Zholkevich (317) showed that the leaves of the same plant lose their synthetic properties at a positive temperature and that growth-inhibiting substances accumulate. According to Tumanov (311) frost hardiness is a property which arises in frost resistant plants only upon termination of growth. The most dangerous period as far as freezing is concerned is when the cells are still increasing in size; the cell division period is one of less sensitivity; and finally the period of nuclear division is still less sensitive to frost (318). To raise the frost resistance of fruit trees, α-naphthyleneacetic acid (319) and maleic hydrazide (320) were applied to slow the growth during the fall and early spring. The results of these tests were quite encouraging. According to other data (321), in many plants short photoperiods slow down growth processes and prepare the plant for winter. Thus, for example, the highest frost resistance is observed in lucerne if grown in the summer with more than an 8 to 12 hr. day; for clover 10 to 14 hr. is sufficient.

Tumanov showed that all plant organs participate in the preparation for winter. The roots are especially important (322). Developing earlier conceptions concerning the frost hardiness of plants and its relation to photosynthesis (312), he found that the accumulation of protective substances in germinating seeds of winter plants is attributable to the transformation of storage substances (311). Sulakadze (323) established that the protoplasm of frost resistant cells possesses a high viscosity and elasticity and can endure intensive dehydration. This result was confirmed by other authors (324), who showed that at temperatures below 0°C., winter corn is highly drought resistant. Riazantsev (325) distinguishes two types of winter transpiration in woody plants; (*a*) stepwise transpiration, that most

frequently encountered, and (*b*) continuously decreasing transpiration. It has been demonstrated that with the approach of frost the movement of water within shoots also completely ceases, and this decreases the loss of water from the surface of the branches (325, 326).

Elucidation of the physiological basis of frost hardiness of various plants and a description of methods of cold protection can be found in the papers of Shutov (327) concerning grape vines and Tumanov (328), who investigated lemons and other citrus trees.

Physiology of Dormancy

Henkel's investigations on the physiology of dormancy in plants, which started in 1948, have been continued. This work closely associates growth phenomena with the problem of wintering. The most important observation made by Henkel is the establishment of the cytophysiological picture of transition of meristematic tissues to a dormant state (329 to 333). This phenomenon consists of the loss of protoplasmic ability to swell, as a result of accumulation of hydrophobic substance of the lipoid type on the plasma surface. Separation of the cell contents then takes place, this being the result of contraction of the protoplasm and sucking in of the plasmodesmata. Oknina & Barskaia (334) and Petrovskaia (333) studied the interaction between protoplasm and nuclei during dormancy. Dormancy is accompanied by changes in the shape and number of nuclei and the accumulation of nuclear substance in the cells which permits the growth processes to commence at a later period. It was demonstrated that these changes take place in the seeds of fruit trees during stratification, and in wintering buds at low temperatures. Delay or premature breaking of preparatory transformations during dormancy hamper further normal growth (335, 336, 337). Kazarian (338) found that even during the vegetative period the protoplasmic separation in cells of lateral nongerminating buds does not disappear. Removal of the terminal buds leads to the establishment of normal protoplasmic relations and to germination of the lateral buds.

Henkel & Sitnikova (339, 340) demonstrated that frost resistance of wintering buds of trees and shrubs depends on the degree of dormancy. Other authors (341, 342) arrived at similar conclusions. A relation between dormancy and protoplasmic separation on the one hand and frost hardiness on the other was also observed in the roots of the apple tree (343) and clover (344). Sharashidze (345), however, could not observe the separation of protoplasm in several evergreen trees in the Caucasus.

In connection with the problem of increasing the frost resistance of plants, a number of authors studied the influence of agricultural methods and of chemical compounds on the dormant state of plants (346 to 349). It was found that dormancy of potato tubers can be terminated by using thiourea, ammonium thiocyanate, rhodanide, and ethylene chlorohydrin; this fact is of practical importance for summer planting of potatoes in the south of the U.S.S.R. (350 to 353). The investigations of Satarova (352,

353) showed that breaking of dormancy in potatoes is accompanied by destruction of the lipoid layers on the protoplasmic surface, by an increase in respiration, by accumulation of free amino acids, and by activation of a large number of enzymes. A practical manual on the diagnosis of dormancy in plants has been written by Henkel & Oknina (354).

Stimulation and Inhibition of Growth Processes

Much attention has been paid to the mode of action and the development of practical applications of growth substances and other synthetic compounds exerting a stimulating, inhibiting, or herbicidal effect on plants. As a result of this work the following has been achieved:

(*a*) The theoretical basis of rapid ripening of fruits under the influence of ethylene, as well as improved methods of practical application of this technique, have been worked out. This work started in 1936 and has recently been reviewed in Rakitin's book (355). It has been shown that the auxins and the ethylene which are formed in fruits are important factors for the biochemical activity of fruits: as long as the fruit tissues are relatively rich in auxins and contain little ethylene, synthetic processes predominate and cell growth takes place. Transition of the fruits from growth to ripening takes place if the tissues contain little auxin and much ethylene; the temporary intensification of metabolic processes, including respiration, which is observed in fruits in the initial period of ripening is attributable to the ethylene formed in the fruit (332).

(*b*) Preparations stimulating the formation of tomato fruits have been developed. The most extensively used is the sodium salt of 2, 4, 5-trichlorophenoxyacetic acid (356).

(*c*) α-naphthylene acetic acid and its compounds, stimulating root formation during vegetative reproduction of plants by grafting, have been tested and are used in practice (357, 358). These preparations have also been used to prevent abscission of ovaries in cotton plants (359) and of apple and pear fruits (360).

(*d*) Substances accelerating potato sprouting by treatment of vegetating plants have been tested (362).

(*e*) Substances inhibiting potato sprouting during storage have been tested and recommended for large scale manufacture. Food potatoes are extensively treated with the methyl ester of α-naphthylene acetic acid (363). The possibility of using chemical preparations to inhibit sprouting in potatoes used for summer planting in the south of U.S.S.R. has also been demonstrated (361). It has been shown that preparations inhibiting the sprouting of potatoes cause weakening of respiration, decrease of the activity of oxidative and some other enzymes, and a general drop in metabolic activity (363). To prevent the growth of shoots in cotton plants, which hampers the maturing of the bolls, it has been suggested that the plant be sprayed with the sodium salt of 2,4,5-trichlorophenoxyacetic acid. This accelerates maturing and noticeably increases the fiber yield (364, 365).

(*f*) To facilitate mechanical harvesting of cotton it has been suggested that magnesium chloride be used for removal of leaves from the plants (366, 367).

(*g*) The sodium salts of 2,4-dichlorophenoxyacetic acid (368, 369) and also the very active butyl ester of 2,4-dichlorophenoxyacetic acid preparation (370, 371) have been proposed as a chemical means of destroying weeds.

Mel'nikov & colleagues have investigated the dependence between structure and physiological action of chemical compounds; the substances mostly studied are those of the phenoxy series (372), substituted phenylacetic and naphthylene acetic acids (373), and also compounds of the isopropyl esters of arylcarbamic acids (374). The distribution of naphthylene acetic acid in plants was studied by labelling the carboxyl group with C^{14}; for the same purpose para-iodophenoxyacetic acid was tagged with I^{113} (375, 376, 377).

Rakitin & Krylov (376) found that naphthylene acetic acid is subject to decarboxylation and other changes in the plant tissues which lead to the loss of the physiological activity of this substance. This phenomenon is regarded as a type of detoxification by which the plant responds to the introduction of abnormal quantities of such substances. Rakitin has also found detoxification of other compounds used to stimulate or inhibit physiological processes or used as herbicides. Substances which are not foreign to the plant (such as indoleacetic acid or ethyl alcohol) may possess an inhibiting or herbicidal action if introduced in the tissues in amounts exceeding the normal; like foreign substances they also are subjected to detoxification (375). It has been demonstrated that physiologically active substances which stimulate growth also intensify the respiration of the plant, change the colloid properties of the protoplasm, and activate many enzymes (375).

The Physiology of Growth

The aim of the plant growth investigations is to determine the nature of the intrinsic processes and the dependence of development on the environment.

Lysenko's stage theory of development (378) has been confirmed during recent years by a number of authors. New proof of the nonreversibility of the vernalization process has been presented by Avakian & Iastreb (379). It was shown, in particular, that high temperatures do not remove the vernalization effect in winter wheat provided that the vernalization interval was sufficient for completion of the stage changes. Nonreversibility of the light stage has been emphasized by Kazarian (380). The return of flowering shoots to vegetative growth was ascribed by this author to nonhomogeneity of cells of the apical point, some of which did not go through the light stage in time and therefore were capable of forming vegetative shoots. Kazarian determined the nonhomogeneity of apical cells by measuring the isoelectric point of their proteins.

Aginian (381) discovered that young embryos of winter wheat undergo vernalization faster than mature embryos. This led the author to the conclusion that wintering properties increase with the age of the seeds. According

to Aginian, young embryos, deprived of the stabilizing influence of the endosperm, easily undergo formative variation. Gunar & Krastina (382) and also Razumov, Oleinikov & Iordanov (383) note the importance of vernalization for the flow of nutrients to the embryo.

As a result of a comparative study of vernalization and the light stage in various sorts of cereals raised in the U.S.S.R. and other countries, Razumov (384) concluded that both stages are adaptive properties which appeared as a result of the given species' inhabiting a definite geographical district. Of interest also in this connection is the work of Dadykin (97), who studied the peculiarities of growth and development of plants grown on cold soil. Moshkov, who has done much work in photoperiodism, concluded that the photoperiodic action in plants first of all causes changes in the physiological activity of the leaves (385). An indirect confirmation of this conclusion may be found in the work of Nichiporovich and co-workers (41, 386, 387), who showed that the direct products of photosynthesis depend on illumination, age, and nutrition of the plant. Chailakhian (388, 389) recently suggested that there is a connection between photoperiodism and photosynthesis. According to this hypothesis, in all plants, irrespective of their photoperiodic reaction, photosynthesis leads to the formation in leaves, among other organs, of some unstable compounds which are necessary for flowering. According to Chailakhian the differences in the photoperiodic response is that in short day plants these products are easily destroyed by light and become stabilized in the dark whereas in long day plants the reaction is the opposite. Minina (77), who worked with maize, cucumber, and pumpkin concluded that leaves are also decisive in determining the sex of flowers. Minina, moreover, found that female sex organs are developed in plants when the redox processes are shifted in the direction of reduction. Britikov & Petropavlovskaia (390), who studied the physiology of inbreeding of winter rye and beet, showed that the treatment of self-pollinated flowers with small amounts of vitamins, enzymes, or auxins considerably raises the probability of fertilization and increases the weight of the seeds.

The grafting method which is frequently applied in physiological experiments in the U.S.S.R. was also used for plant development investigations. Efeikin (391), Kraievoi (392), Avakian (393), Kruzhilin & Shvedskaia (394, 395), and others showed that seedlings of biennial plants grafted to the same plants in the second year, or to annual forms, may flower and bear fruit during the first year.

The problem of "wholeness" of plants and the interaction of various organs has attracted the attention of plant physiologists. Previously the relation between leaves and stem buds was the problem mostly studied, but now, after the discovery of the synthetic function of roots, the interaction between roots and other organs has also been receiving attention. The most detailed study of this problem has been made by Chailakhian (396). Polarity in plants has also been investigated from this viewpoint. Molotkovski (397, 398, 399) showed that various substances located in the axial organs of the plant have definite physiological gradients. By varying the position

of the organs with respect to the force of gravity, Molotkovski observed in them a redistribution of ascorbic acid and some other substances. It was possible in this manner to terminate prematurely the dormant state in oak, walnut, and linden tree branches and to inhibit the formation of flower organs in red perilla and kenaf (*Hibiscus cannabinus*). Finally, by ringing and applying indoleacetic acid to lemon cuttings, Chailakhian & Nekrasova (400) were able to overcome their polarity, causing roots to be formed on the apical end of the stem and new shoots at the base.

Modern techniques and new research methods, the clarity of the problems to be solved, and broad perspectives in the future have attracted many scientists to plant physiology during recent years. This permits one to hope that the study of the life of plants will meet with new success in the next few years. In this large and joint task scientists of the U.S.S.R. hope to make their contribution.

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AUTHOR INDEX

A

Abe, S., 71
Abelson, P. H., 5
Åberg, B., 237, 239, 250, 251, 252, 254, 257, 258, 259, 260, 374, 377, 381
Abidin, Zain ul, see Zain ul Abidin
Abramsky, M., 283
Adam, N. K., 357, 365
Addicott, F. T., 368
Agakishev, A., 416
Aginian, A. A., 420
Aichele, F., 299
Ainsworth, C. C., 115
Ainsworth, G. C., 156
Akamine, E. K., 302, 306
Akazawa, T., 283, 284
Akimochkina, T. A., 404, 410, 416
Albaum, H. G., 244, 325, 334
Albers, W., 325
Aleev, B. S., 66
Alekseev, A. M., 414, 415
Alfert, M., 94
Alivisatos, S. G. A., 284
Allard, H. A., 156, 191, 192, 193, 194, 197, 208
Allen, E. K., 51
Allen M. B., 29, 37, 39, 44, 54, 55, 56, 57, 63, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 349, 351, 352
Allen, O. N., 51
Allen, P. J., 117, 120, 129, 134
Allen, S. E., 256
Allfrey, V. G., 95, 96, 103
Allington, W. B., 118
Allison, F. E., 54, 56, 57
Allison, R. K., 54, 55
Allyn, W. P., 176
Alpat'ev, A. M., 414
Alton, C. H., 135
Anderson, D. G., 270
Anderson, J. A., 120
Anderson, L., 343
Anderson, N. G., 360
Anderson, P. G., 29
Andeweg, J. M., 391
Andreae, S. R., 247
Andreae, W. A., 247
Andreeva N. A., 413
Andreeva, T. F., 403
Andrewes, C. H., 129
Anisimov, A. A., 404
Anker, W., 248
Anscheutz, I., 3
Applewhite, T. H., 17
Archer, J. R., 359
Arens, K., 118, 120
Areshkina, L. Ia., 412
Arisz, W. H., 7, 11, 13, 17
Arnal, L., 72
Arnold, W., 36, 43, 46
Arnon, D. I., 325-54; 29, 37, 39, 44, 55, 56, 57, 177, 269, 290, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 349, 351, 352
Aronescu-Savulescu, A., 134
Aronoff, S., 15, 329, 337, 365
Arthur, J. C., 127, 128
Arthur, W., 47
Artsikhovskaia, E. V., 408
Aschoff, J., 84, 85
Aseieva, K. V., 411
Ashby, E., 85
Ashby, W. C., 238, 254, 256
Ashcroft, R. T., 185
Ashkinazi, M. S., 403
Ashwell, G., 291, 292
Atchison, E., 259
Atkinson, D. E., 60
Atkinson, T. G., 120
Atterburg, A., 310
Auchincloss, R., 286
Audus, L. J., 237, 239, 242, 246, 254, 256,257, 258, 259, 260, 261, 363
Avakian, A. A., 420, 421
Avery, G. S., 195, 205
Avery, G. S., Jr., 300
Axelrod, B., 267-98; 268, 269, 271, 272, 273, 284, 288, 291, 338
Ayengar, P., 284
Ayland, M. J., 3, 10

B

Baar, H., 316
Babushkin, L. N., 403
Bach, M. K., 63
Bagaiev, V. B., 405
Bail, O., 129
Bailey, D. L., 133, 134
Bailey, T. W., 98, 102, 103
Baillaud, L., 72
Baird, E., 198
Baker, E., 355, 359, 365
Baker, E. G. S., 120
Baker, J. A., 118
Baker, R. R., 145
Baker, R. S., 394
Bakhir, A. I., 410
Baldovinos, G., 238, 255
Baldwin, D. M., 129
Baldwin, I. L., 124
Ball, C. D., 230
Ball, N. G., 75, 77
Bandurski, R. S., 257, 258, 268, 269, 271, 272, 273, 338
Banerjee, B., 307
Baraccio, A., 318
Baranova, V. Z., 412
Bardinskaia, M. S., 412
Barer, R., 44
Barkadye, T. G., 178
Barker, J., 268, 282
Barltrop, J. A., 36, 42
Barner, D., 108
Barnett, H. L., 123
Barnett, R. C., 272
Barnum, C. P., 96
Barrenscheen, H. K., 325
Barrett, J. T., 135
Barron, E. S. G., 107, 268, 279
Barrus, M. F., 127
Barskaia, E. I., 418
Bartley, W., 13
Barton, L. V., 299, 302
Baskakov, I. A., 420
Baslavskaya, S. S., 404
Bassham, J. A., 26, 27, 28, 40, 43, 276, 286, 290, 347
Bastin, E. S., 134
Bastin, R., 301
Bauer, E. W., 86
Bawden, F. C., 123, 124, 152, 162
Baxter, P., 180
Bayles, B. B., 232
Beadle, G. W., 123
Beament, J. W. L., 362
Beatty, R. A., 99
Becker, R. S., 32, 42
Becker, T., 79
Beemster, A. B. R., 377, 378, 379, 383, 391, 395, 397
Beevers, H., 267-98; 276, 278, 280, 281, 283, 284, 362
Begishev, A. N., 404
Beijerinck, M. W., 51, 129
Bein, M. L., 240
Belcher, R., 180
Belikov, P. S., 412
Benitez, A., 44, 46
Bennet-Clark, T. A., 249
Bennett, C. W., 143-70; 143, 145, 146, 147, 151, 154, 155, 156, 158, 161, 162, 163, 166
Bennett, F. T., 127
Bennett, J. P., 177, 178, 182
Bennett, S. H., 364, 366
Benson, A. A., 26, 27, 28, 44, 276, 286, 287, 288, 290, 334, 337, 347
Bentley, J. A., 252, 362, 366
Beraha, L., 144
Berezovskii, M. I., 420

Berkeley, M. J.,116
Berks, R., 123
Berling, H. H., see Hoffman-Berling, H.
Berman, M. D., 5
Bernard, N., 123, 125, 128
Bertani, G., 123
Berzin'sh, A., 419
Bessey, C. E., 134
Bessey, E. A., 129
Besson, G., 240,259
Biale, J. B., 283
Bickle, A.S., 252,366
Biddulph, O., 178,180,181, 364
Biddulph, S., 365
Bisby, G. R., 115
Bishop, J. R., 237
Bisset, K. A., 99
Bitancourt, A., 145,261
Bittner, J. J., 84
Blaauw-Jansen, G., 396
Black, L. M., 121, 127, 143
Black, M., 84, 202, 203, 206, 317
Blackman, G. E., 360, 361, 363
Blagoveschchenskii, A. V., 306
Blair, B., 361
Blanchard, K. C., 95
Blinks, L. R., 33, 34, 40, 55, 62
Blish, M. J., 217
Blondeau, R., 358, 359, 361, 363, 366, 368
Bloom, B., 280, 282
Bogdanov, P. L., 192, 194
Böhmer, K., 317
Boiarkin, A. N., 420
Boichenko, E. A., 403
Boiko, L. A., 416
Bokarev, K. S., 420
Bold, H. C., 98
Boll, W. G., 240, 248
Bolle-Jones, E. W., 181
Bolotina, T. T., 409
Bolton, E. T., 5
Bonar, L., 123
Bond, G., 64, 65
Bond, T. E. T., 128
Bonner, J., 2, 3, 4, 17, 82, 110, 198, 203, 204, 205, 215, 244, 257, 258, 268, 269, 283, 338, 360, 387
Bonner, W. D., Jr., 307, 309
Booij, H. L., 363
Boone, D. M., 124
Boothroyd, C. W., 117
Boothroyd, R. E., 99
Borodulina, A., 405
Bortels, H., 55, 64, 65
Borthwick, H. A., 299-324; 84, 198, 202 203, 204, 301, 303, 307, 311, 313,314, 315, 316, 374, 377, 382, 383, 384, 385, 386, 391, 394, 395, 397
Bosemark, N. O., 251
Boss, J., 102
Bottelier, H. P., 393
Bouillenne, R., 391
Bovet, P., 95
Bowler, E., 355, 359, 365
Boyer, P. D., 29
Boynton, D., 364
Bracher, R., 85
Brachet, J., 94, 95, 97, 101, 103, 104, 105, 106, 107
Bracket, F. S., 40
Bradley, D. F., 43, 286, 335, 336, 337
Bradley, R. H. E., 149
Braun, A. C., 109
Braunstein, A. E., 410
Breed, R. S., 122
Brefeld, O., 116, 120, 130
Bregetova, L. G., 420
Bregoff, H. M., 37, 53, 58, 59, 60
Bretz, T. W., 155
Brian, R. C., 361
Brierly, W. B., 115
Briffaz, M., 308
Briggs, D. R., 229
Brin, G. P., 402
Bristol, B. M., 53
Britikov, E. A., 421
Britten, R. J., 5
Broadbent, D., 241, 246
Broadbent, L., 151
Brodia, A. F., 271
Brohult, S., 215
Brooks, S. C., 6, 7,
Brouwer, R., 17, 18, 19
Brown, A., 283
Brown, A. H., 15, 43, 325, 335
Brown, A. S., 278
Brown, E., 302, 311
Brown, F. A., 84, 85
Brown, J. C., 171-90; 174, 175, 176, 177, 178, 181, 182, 183, 184, 185, 186
Brown, J. W., 179, 180, 357, 359, 364, 365
Brown, R., 1, 238, 239, 240, 241, 246, 303, 309
Brown, S. A., 230, 289
Brown, W., 115,117,124,126, 127, 129
Broyer, T. C., 18, 19
Brumfield, R. T., 238, 368
Brummond, D. O., 283
Bruns, V. F., 302
Buchanan, J. G., 286
Bucher, O., 99
Buchholz, W., 40
Budagovski, V. I., 418
Bühnemann, F., 74, 76, 77, 80, 81
Bukin, V. N., 413
Buller, A. H. R., 116
Bullough, W. S., 105,106, 109, 110
Bundel, A. A., 222, 224, 411
Bünning, E., 71-90; 73, 75, 76, 77, 79, 82, 83, 84, 86, 193, 313
Bünsow, R., 75, 80, 83, 84
Burdetto, E. W., 43
Burk, D., 9, 40, 41, 56
Burkholder, P. R., 195, 205, 374
Burns, G. R., 388
Burris, R. H., 51, 52, 53. 55, 57, 59, 60, 61, 66, 67, 252, 283, 347
Burström, H., 11, 12, 237, 238, 243, 245, 248, 249, 251, 253, 256, 258, 259, 260, 261, 262, 306, 318
Burtch, L. M., 178, 183
Burton, K., 41
Burygin, V. A., 416
Busch, G., 77
Büsgen, M., 195
Butler, E. J., 115, 122, 124, 125, 127, 128, 131, 135
Butler, G. W., 6, 14
Butlin, K. R., 67
Butts, J. S., 356, 358, 359
Byerrum, R. U., 230

C

Cabib, E., 287
Cailachjan, M. C., 198, 205
Cain, J. C., 181
Cal'chenko, I. N., 410
Caldwell, J., 144, 153, 163
Calhoun, H. G., 118, 135
Calhoun, J. B., 84
Calvin, M., 26, 27, 28, 30, 32, 36, 40, 42, 43, 44, 62, 184, 276, 283, 286, 287, 290, 329, 334, 335, 336, 337, 347
Campbell, G. F., 217, 221, 229, 231
Canillot, R., 258
Cannon, W. A., 128, 135
Capindale, J. B., 29, 44, 340, 342, 343, 344, 345, 349, 351
Capoor, S. P., 154, 161, 162
Caputto, R., 286
Cardini, C. E., 286, 287
Carleton, M. A., 133, 134
Carlson, C. W., 184
Carns, H. R., 368
Carpenter, J. B., 131
Carr, D. J., 206
Carr, P. H., 355
Carrero, J. O., 176
Cartwright, P. M., 241
Caspary, R., 311, 312
Castor, L., 45
Cation, D., 155
Caullery, M. J. G. C., 135
Cavallito, C. J., 309
Cavazza, L., 304
Cervantes, J., 127
Cervigni, T., 318

Chaet, A. B., 110
Chailakhian, M. Kh., 405, 407, 414, 421, 422
Chamberlain, D. B., 118
Chance, B., 40, 45
Charalampous, F. C., 288
Charles, A., 4
Chaudhri, I. I., 84
Chayen, J., 106
Cheadle, V. I., 365
Chefurka, W., 279
Chen, Y. T., 293, 294, 295
Cheng, L. T., 229
Cheo, P. C., 156
Chepil, W. S., 302
Chester, K. S., 128, 133, 135
Chetverikova, E. P., 408
Chèvremont, M., 98, 103
Chiba, Y., 36
Chibnall, A. C., 216, 357
Chiribogo, J., 287
Chouard, P., 192, 194, 197, 209
Christensen, C. M., 123
Christensen, J. J., 123, 124, 127
Christian, W., 269
Church, A. B., 45
Clagett, C. O., 309
Clark, T. A., see Bennet-Clark, T. A.,
Clark, W. G., 368
Clarke, H. T., 1
Clausing, C. A., 108
Clauss, H., 80, 254
Clayton, R. K., 33, 341
Cleveland, L. R., 118
Clinch, P. E. M., 155, 156
Cline, D. M., 124
Clinton, G. P., 119, 131
Cloudsley-Thompson, J. L., 84
Cochran, G. W., 147
Cochran, L. C., 150, 152, 154, 155, 159, 160
Cockerham, G., 152
Cohen, A. L., 130
Cohen, S. S., 108, 279, 282
Cole, C. V., 180
Collander, R., 363
Colley, R. H., 126
Collyer, D. M., 53, 57
Colvin, J. R., 250
Commoner, B., 244
Conn, E. E., 270, 272, 284, 317, 318
Conover, J. R., 357, 359, 364
Conway, E. J., 5, 11, 15, 16
Cooil, B. J., 244, 255
Cook, J., 364
Coons, G. H., 120
Cooper, D. C., 95, 124
Cooper, D. R., 231
Corbett, M. K., 151
Cori, O., 271
Cormack, R. G. H., 238
Cormier, M. J., 47
Corner, E. J. H., 127
Cory, R. A., 365
Costa, A. S., 158, 166
Couch, H. B., 156, 157
Couch, J. N., 118
Cowie, D. B., 5, 6, 7
Cowie, L., 279
Cox, L. G., 306
Cox, V. J., 74
Crabill, C. H., 134
Crafts, A. S., 143, 164, 165, 359, 362
Craven, P. M., 232
Creighton, H. B., 195, 205
Crick, F. H. C., 96
Crickard, R. G., 40
Crinquand, E., 72
Crocker, W., 299, 301, 302, 312, 375
Crooks, W. M., 183
Cross, F. B., 179
Csonka, F. A., 229
Cummins, G. B., 127, 128
Cuninghame, R. C. R., see Robertson-Cuninghame, R.C.
Currier, H. B., 365
Curtis, J. J., 232
Curtis, O. F., 165
Cutolo, E., 288
Cutter, V. M., 117, 120, 123, 134

D

Dadykin, V. P., 405, 421
Dagis, I. K., 413
Dain, B. Y., 403
Dalberg, L. Y., 79, 252, 261
Daly, J. M., 123
Daly, M. M., 95, 96
Dam, H., 341
Damaschke, K., 37
D'Amato, F., 307
Daniel, T. W., 77
Danielli, J. F., 1, 359
Daniels, F., 40, 43
Danielson, C. E., 215-236, 216, 218, 219, 220, 221, 222, 223, 224, 225, 226, 233
Daoust, R., 96
Daran, D. V., 120, 132
Darlington, H. T., 302
Daubenmire, R. F., 208
Daus, L. L., 286
Davenport, H. E., 43, 342
Davidson, J. B., 43
Davidson, J. N., 95, 96, 104
Davies, R., 123
Davies, R. E., 5, 13
Davis, B. D., 289
Davison, D. C., 318
Day, B. E., 365
De, P. K., 57, 65, 66
Dean, L. A., 180
DeBary, A., 115, 116, 122, 126, 135
DeBruyn, H. L. G., 123
De Kock, P. C., 179, 181, 183, 306
de la Haba, G., see Haba, G. de la
DeLamater, E. D., 99
de Ley, J., see Ley, J. de
de Mouros, M. A., see Mouros, M. A. de,
den Honert, T. H. van, see Honert, T. H. van den
der Hart, J. M. van, see Hart, J. M. van der
de Ropp, R. S. see Ropp, R. S. de
DeRose, H. R., 358, 363
der Scheer, C. van, see Scheer, C.van der
Desjardins, P. R., 155, 156
Deysson, M., 256
Diachun, S., 144
Dickens, F., 269, 270, 291
Dickinson, S., 117, 118, 119, 120, 127, 131, 132
Dignadice, P. B., 135
Dische, Z., 272
Distler, J., 301
Dobrovolskaia, N. N., 413
Doerschuk, A. P., 293
Doidge, E. M., 133
D'Oliveira, B., 134
D'Oliveira, M. de L., 131
Doman, N. G., 403
Doniach, A. H., 95
Doolittle, S. P., 155
Doorenbos, J., 192, 211
Döring, H., 77, 78
Dorough, G. D., 32
Doudoroff, M., 286, 292
Dougherty, E. C., 116, 118, 120, 135
Douin, R., 52, 54, 64
Down, E. E., 157
Downey, M., 5
Downs, R. J., 313, 384, 397
Drake, V. C., 311
Drewes, K., 51, 53, 65
Drouineau, G., 181
Drozdova, T. V., 411
Drumm, K., 396
Dubinina, I. M., 407, 408
Dubos, R. J., 101, 120
Du Buy, H. G., 368
Duggar, B. M., 33, 116, 119, 126, 143, 159
Dugger, W. M., Jr., 366
Duhamet, L., 110
Dunham, R. S. 308
Dunn, A., 110
Durham, L. J., 343
Durmishidze, S. V., 413
Durrell, L. W., 133
Dushechkin, A. I., 413
Dutton, H. J., 33
Duysens, L. N. M., 25-50;33 34, 35, 37, 38, 40, 41, 44, 45, 342, 351
Dyke, I. J., 75, 77

E

Earl, J. M., 279
Earle, F. R., 232
Edelman, J., 281
Edelstein, M. M., 405
Edwards, M., 309
Edwards, T. I., 310
Efeikin, A. K., 421
Ehrenberg, M., 77
Eichin, R., 240, 248
Elenev, L. K., 192
Eliasson, L., 246
Elliott, B. B., 308, 309
Elliott, C., 129
El Mangouri, H. A., 357
El Saifi, A. F., 268
Elsden, S. R., 58
Elstad, V., 313, 375, 376, 386, 390
Elvehjem, C. A., 173
Emerson, R., 36, 41, 116, 130
Emerson, R. L., 327, 328, 329, 330, 342
Enderle, W., 75
Engel, H., 77
Engle, H. B., 66
English, J., Jr., 110
Enklewitz, M., 292
Entner, N., 292
Ephrussi, B., 93
Eppeley, R. W., 45
Eppling, F. J., 52, 53
Epstein, E., 1-24; 2, 3, 4, 6, 8, 9, 10, 11, 12, 14, 16, 18, 19
Eremenko, V. E., 415
Erickson, R. O., 95, 100, 103, 104, 109, 242
Erofeiva, N. N., 413
Esau, K., 143, 149, 153, 154, 155, 156, 159, 161, 356, 358, 359, 365
Euler, H. von, 287
Evans, H. J., 14
Evans, R. J., 232
Evans, R. W., 40, 43
Evenari, M., 205, 299, 301, 303, 306, 308, 309, 312,313, 314, 315, 316
Everett, H. L., 117
Everson, L. E., 308
Evstigneev, V. B., 31, 402
Evstigneieva, Z. G., 411
Eyring, H., 31, 34, 36, 41, 43, 44
Eyster, C., 56
Ezekiel, W. N., 120, 129, 133

F

Fager, E. W., 334, 337
Falcone, G., 107
Famintsin, A. S., 401
Fang, S. C., 364, 366
Fassuliotis, G., 120
Fauré-Fremiet, E., 85
Fawcett, H. S., 145
Fawzy, H., 12
Fergerson, M. S., 118
Ferrari, G., 47
Fife, J. M., 167
Filippovich, I. I., 409
Fingerman, M., 84, 85
Fink, B., 116, 135
Fink, E., 240, 259
Fischer, H., 396
Fisher, E. G., 360, 364
Fitzgerald, G. P., 54
Fitzpatrick, H. M., 124, 131
Flemion, F., 312
Flint, L. H., 313, 316, 377, 382, 395
Flügel, A., 73, 75
Fogg, G. E., 51-70; 51, 52, 53, 54, 55, 57, 58, 61, 63, 65, 66, 357, 358
Fontaine, T. D., 227
Forrest, R. S., 291
Förster, T., 34
Fortanier, E. J., 379, 381, 395
Fortini, S., 318
Fouarge, M., 391
Fowden, L., 66
Frampton, V. L., 167
Franck, J., 37, 41, 343
Frank, B., 51
Fraps, R. M., 84
Fred, E. B., 124
Frederic, J., 98, 103
Freed, V. H., 356, 358, 359, 366
Fremiet, E. F., see Fauré-Fremiet, E.
French, C. S., 33, 45, 46, 380, 388, 389
Frenkel, A. W., 37, 40, 60
Frey-Wyssling, A., 4
Frick, L., 123
Friederichsen, I., 77
Fries, N., 248, 255
Fritsch, F. E., 64, 65
Fritz, G., 283, 284, 300
Fuerst, C. R., 230
Fujii, R., 318
Fuller, H. W., 361
Fuller, R. C., 30, 62,290
Fulton, R. W., 163
Funahashi, S., 283
Funke, G. L., 374, 375, 377, 379

G

Gabrielsen, E. K., 388
Gaffron, H., 37, 328, 334, 337, 342
Gaffron, J., 40
Gager, C. S., 116
Gale, E. F., 123
Galinskaia, M. S., 415
Galston, A. W., 79, 252, 261, 367, 394, 395
Garber, E. O., 123
Gardner, M. W., 155
Garner, J. M., 120
Garner, W. W., 191, 192, 193, 194
Garrard, A., 251, 254
Garton, G., 363
Gassner, G., 309, 316
Gattiker, R., 99
Gauch, H. G., 179, 366
Gaumann, E., 115, 127, 129
Gautheret, R. J., 86, 108
Gavrilova, V. A., 31, 402
Genderen, H. van, 30
Geoffrey, H. B., 173
Gerloff, G. C., 54, 56
Gessner, F., 3
Gest, H., 37, 52, 53, 58, 59, 60, 329
Gevorkiantz, S. R., 191
Ghiretti, F., 279
Gibbs, M., 269, 270, 274, 277, 279, 280, 281, 282, 283,
Gibson, D. M., 284
Gibson, G. W., 130
Giddings, N. J., 146
Gilbert, W. W., 155
Gilder, H., 172
Gile, P. L., 183
Gilpatrick, J. D., 128
Ginsburg, V., 279, 281, 285
Girnik, D. V., 418
Girth, H. B., Jr., 116
Glaser, R. W., 116
Glavind, J., 341
Glenister, P. R., 176
Glikman, T. S., 403
Gloor, H., 100, 108
Glover, J., 30
Goddard, D. R., 109, 129, 172, 242, 283, 317
Godnev, T. N., 403, 406
Goedheer, J. C., 46
Gold, A. H., 155, 157
Goldschmidt, E. P., 362
Goldsworthy, M. C., 129
Göllner, E., 77, 86
Golovin, P. V., 410
Good, N., 43, 44
Goodey, T., 135
Goodman, M., 286, 335, 336, 337
Goodwin, R. H., 109, 238, 239, 242, 243, 247, 385, 386
Gordon, S. A., 257
Gordon, S. H., 361
Gorev, L. N., 416
Gorter, C. J., 194, 198, 258
Gortner, W. A., 367
Gottlieb, D., 120, 121
Gouwentak, C. A. R., see Reinders-Gouwentak, C. A.
Graf, G. E., 15
Graf-Marin, A., 132
Graillot, G., 318
Grainger, J., 129

Granick, S., 172, 173
Grant, V. Q., 237
Greathouse, G. A., 285
Green, D. E., 61
Green, M., 60
Green, R. J., 120
Greene, S., 239, 243, 247
Greenwood, A. D., 309
Gregory, F. G., 79, 367
Gregory, P. H., 130
Greiner, C. M., 269, 271, 272, 273
Gretschushnikoff, A. I., 129
Greulach, V. A., 259
Griese, A., 269
Griffiths, M., 47
Grinenko, V. V., 419
Grinfeld, E. G., 404
Grob, E. C., 240, 248
Grohne, U., 301
Grossenbacher, K. A., 77
Grube, K. H., 331, 333
Guérillot, J., 318
Guérillot-Vinet, J., 318
Guernsey, F. S., 251, 301, 367, 392
Gulisashvili, V. Z., 192, 193, 196, 197
Guminskaia, M. A., 405
Gunar, I. I., 420, 421
Gunar, V. I., 411
Gurnsey, H. E. F., 115, 116, 122, 126, 135
Gusev, N. A., 414, 415
Gustafson, F. G., 196
Guttenberg, H. von, 367

H

Haagen-Smit, A. J., 110
Haans, A. J. M., 44
Haba, G. de la, 272, 273
Hackett, A. J., 123
Hackett, D. P., 14, 283
Haddock, J. C., 232
Hagan, R. M., 77
Hageman, R. H., 179, 269, 300
Hagen, C. E., 4, 9, 10, 11, 16, 19, 84, 177, 301, 364
Hagen, C. R., 309
Halberg, F., 84
Haldane, J. B. S., 97
Hale, C. M. F., 99
Hall, A. G., 276
Hall, R. P., 122
Hamada, M., 122
Hamly, D. H., 304
Hamlyn, B. M. G., 149, 152
Hammarlund, C., 127, 133
Hammond, M. R., 95
Hamner, C. L., 361
Hamner, K. C., 82, 198, 200, 203, 206, 355, 359, 365
Hancock, C. R., 367
Handley, R., 4, 12
Hansch, C., 257, 363
Hansen, B. A. M., 241, 257, 258
Hansen, H. N., 120
Hanson, J. B., 2, 3, 4, 9, 10, 17, 81, 300, 360
Hanson, V., 360
Hapton, C. E. L., 365
Harari, I., 30
Harder, R., 64, 77
Harley, C. P., 179, 181
Harrington, G. T., 311
Harris, A. Z., 26, 27, 28, 290, 347
Harris, I. F., 221
Harrison, J. A., 2, 16, 17
Harrison, R. W., 130, 135
Hart, H., 121, 126
Hart, J. M. van der, 35
Hartman, E. L., 179
Hartman, R. J., 229
Hartt, C. E., 286
Haskell, T. H., 309
Hassan, M. N., 13
Hassid, W. Z., 53, 279, 281, 285, 286, 288
Hauser, E. W., 361, 366
Hawk, E. A., 120
Hawker, L. E., 117, 127
Haxo, F., 33
Hay, J. R., 366
Hayes, P. M., 36, 42, 286, 287
Hayward, H. R., 8, 11
Heald, F. D., 116, 122
Heald, W. R., 11
Hedges, F., 130
Heilbrunn, L. V., 110
Heimann, M., 77
Heinricher, E., 312
Heinze, P. H., 313, 384
Helgeson, E. A., 309
Heller, V. G., 179
Henderson, J. H. M., 244
Henderson, R. G., 156
Hendricks, S. B., 299-324; 19, 84, 175, 177, 202, 203, 204, 301, 307, 311, 313, 314, 315, 316, 374, 377, 382, 383, 384, 385, 386, 394, 395, 397
Henkel, P. A., 414, 415, 416, 417, 418, 419
Henkes, H. E., 84
Henriksson, E., 57, 64, 65
Henssen, A., 86
Hérisset, A., 52, 62, 65
Hervey, A., 122
Hess, C., 79
Hesse, O., 310
Hewitt, E. J., 181, 182, 183
Hewitt, W. B., 149, 153, 159
Hiatt, H. H., 289
Hickman, C. J., 131
Hickman, J., 291, 292
Hidalgo, J., 186
Higgins, B. B., 124
Highkin, H. R., 81
Higinbotham, N., 2, 3, 9, 10, 360
Hill, H. H., 232
Hill, R., 43, 44, 342
Hirota, K., 358
Hirsch, H. E., 276
Hirschfeld, A., 334
Hitchens, A. H., 122
Hitier, H., 126
Hoagland, D. R., 2, 19
Hobby, G., 40, 41
Hochster, R. M., 291
Hoff-Jørgensen, E., 95
Hoffman, D., 210
Hoffman-Berling, H., 102
Höfler, K., 6
Hofmann, A. W., 305
Hofmann, K., 85
Hogan, G. L., 54, 55
Hogeboom, G. H., 1
Hogue, M. J., 99
Hok, K. A., 118, 120, 123, 130
Holden, C., 95
Holley, R. W., 181
Hollowell, E. A., 311
Holmes, F. O., 122, 123, 128, 129, 151, 158, 162
Holmes, R. S., 175, 176, 177, 178, 181, 182, 183, 184, 185, 186
Holm-Hansen, O., 56
Holt, A. S., 31, 44, 380, 388, 389
Holzer, H., 336, 338, 347
Honda, S. I., 10, 13, 14, 17
Honert, T. H. van den, 7, 19, 361, 362, 365, 368
Honing, J. A., 304, 305
Hoover, S. R., 54, 56, 57
Hoover, W. H., 388
Hooymans, J. J. M., 19, 361, 362
Hope, A. B., 4, 5, 6, 14
Hopkins, E. F., 183
Hopkins, F. G., 317, 318
Hopkins, H. T., 9, 11, 19, 243, 244, 245, 364
Horecker, B. L., 27, 28, 270, 272, 273, 274, 277, 279, 281, 288, 289, 290
Horn, M. J., 227
Horne, V., 361, 363, 368
Horner, C. K., 56
Horning, E. S., 300
Horowitz, H. H., 293
Horsfall, J. G., 363
Hotchkiss, R. D., 96
Hotson, H. H., 118, 123, 134
Hough, L., 291
Housley, S., 362
Houston, B. R. 149, 153, 157, 159
Houwink, A. L., 359
Howell, R. T., 249
Hoyle, D. A., 192, 194, 198, 201
Hughes, A., 100, 101, 102, 103, 106
Hughes, J. H., 308

Huiskamp, W. J., 35
Humphrey, G. G., 96
Hunter, J., 40, 41
Hunter, J. G., 182, 183
Hunter, R. F., 306
Hurd-Karrer, A. M., 125
Hurst, H., 357, 359
Huseby, R. A., 96
Huskins, C. L., 94
Hussey, G., 84
Hutcheson, R. M., 292
Hutchins, L. M., 150, 152, 153, 154, 159, 160
Hutchinson, G. E., 66
Hutner, S. H., 58, 334
Hyde, E. O. C., 304
Hyde, T. G., 234
Hylmö, B., 4, 6, 18, 19

I

Iakusheva, E. I., 417
Iakushkin, I. V., 405
Iastreb, M. G., 420
Iermakov, A. I., 410
Il'in, G. S., 405, 407, 412
Iljin, W. S., 176, 178, 181
Inaoka, M., 14
Ingestad, T., 363
Ingold, C. T., 74
Inkson, R. H. E., 183
Iordanov, I. T., 421
Irving, G. W., 227
Isaieva, E. S., 409
Isherwood, F. A., 293, 294, 295
Ishii, M., 129
Isikawa, S., 84, 313, 317
Ivanitskaia, E. F., 416
Ivanova, G. V., 410
Ivanova, T. M., 408
Iwamura, I., 81
Iwanowski, D., 129
Izard, C., 126

J

Jackel, S. S., 293
Jackson, H. S., 123
Jacobs, E. E., 31, 44
Jacobs, W. P., 367, 368
Jacobson, L., 3, 4, 12, 13, 15, 176, 177, 184
Jacobson, W., 104
Jaeger, C. M., 232
Jagendorf, A. T., 215, 255
Jakob, J. G., 131
James, W. O., 7, 172
Jang, R., 269, 271, 272, 291
Jansen, G. B., see Blaauw-Jansen, G.,
Jauregg, T. W., see Wagner-Jauregg, T.
Jaworski, E. G., 364, 366
Jeffers, W. F., 123
Jemukhadze, K. M., 412
Jensen, J. H., 123
Jensen, R., 220
Jensen, W. A., 105, 110, 242, 246
Jerchel, D., 305
Jester, J. R., 192
Johns, C. O., 227
Johnson, A. W., 309
Johnson, B., 143
Johnson, E. M., 144
Johnson, F., 146
Johnson, L. P. V., 302
Johnson, M. P., 313, 360
Johnson, P., 227, 228
Johnson, W. H., 120
Johnsson, H., 192
Johnstone, K. H., 120
Jones, D. B., 227, 229
Jones, E. W. B., see Bolle-Jones, E. W.,
Jones, H., 75
Jones, J. K. N., 291
Jones, L. H., 183
Jones, M. B., 313, 314
Jones, P. M., 130
Jones, S. G., 115, 124, 125, 127, 128, 131, 135
Jones, V. V., 177
Jönsson, E., 239, 252
Joubert, F. J., 226, 228, 231

K

Kachan, A. A., 403
Kaden, S. B., 409
Kajima, M., 234
Kalckar, H. M., 288, 225
Kalinin, F. L., 415
Kallio, P., 81
Kalmus, H., 84
Kalnin'sh, A. I., 419
Kamen, M. D., 30, 37, 40, 44, 52, 53, 58, 59, 60, 329
Kamien, E. N., see Niedergang-Kamien, E.
Kamiya, N., 71, 72
Kandler, O., 238, 240, 244, 252, 254, 255, 256, 258, 259, 330, 332, 334, 335, 348, 349, 350, 351
Kaplan, N., 286
Karling, J. S., 130
Karlsson, B., 246
Karschon, R. von, 211
Karstens, W. K. H., 108, 367
Kasha, M., 32, 42
Kassanis, B., 158
Katagiri, M., 289
Katalymov, 406
Katznelson, H., 7
Kaufman, S., 284
Kaufmann, E., 306
Kausche, G. A., 156
Kawaguchi, S., 276, 287
Kay, E. R. M., 96
Kay, L. D., 26, 27, 28, 290, 347
Kazarian, V. O., 418, 420
Kearns, V., 311
Keay, M. S., 119, 120, 131
Kedrov-Zikhman, O. K., 406
Keener, P. D., 135
Kefford, N. P., 249
Keitt, G. W., 123, 124
Kelman, A., 123
Kendrick, J. B., 155
Kent, G. W., 122, 135
Kent, M., 367
Kern, F. D., 117, 122, 125, 128
Kersten, J. A. H., 33
Keyes, C. G., 198
Kholodnyi, N. G., 401, 417
Khudairi, A., 200
Khudiakova, R. Y., 403
Kidder, G. W., 173
Kihara, H. K., 96
Kimura, J., 183
King, C. G., 293
King, H., 129
Kinzel, W., 313
Kipp, M., 301, 317
Kirillov, G. N., 413
Kirk, P. L., 103, 104
Kirsanov, A. T., 178
Kitching, J. A., 1
Klas, Z., 57, 66
Klebs, G., 198
Klechkovskii, V. M., 405
Klein, R. M., 268
Klein, S., 301
Klein, W. H., 313, 375, 376, 386, 390
Kleinhoonte, A., 74, 75, 76
Klenow, H., 272, 273, 288
Kleshnin, A. F., 381, 404, 416
Klevestrand, R., 291
Kliman, S., 174
Klimenko, V. G., 232
Kmetec, E., 283, 300
Knapp, R., 7
Knauss, H. J., 9
Kobiakova, A. M., 409, 414
Koblents-Mishke, O. I., 404
Kobzeva, A. A., 409
Koch, A. L., 96
Kocherzhenko, N. G., 417
Koffer, H., 362
Kok, B., 37, 40, 43, 340, 350, 351
Kolesnik, F. I., 413
Kolesnikov, P. A., 408, 411
Kolesnikova, N. V., 410
Koller, D., 302
Kolomiets, P. T., 418
Kolosov, I. I., 405, 406, 407, 411
Konarev, V. G., 411
Kondo, K., 234
Kondrasheva, A. A., 413
Konishi, C., 66
Koontz, H. V., 365
Kopp, M., 246
Korey, S. R., 30

Kosar, W. F., 308
Kosobutskaia, L. M., 403
Kostychev, S. P., 401
Kotel'nikova, A. V., 268, 269, 409
Kovachevich, R., 292
Kovda, V. A., 416
Kraievoi, S. I., 421
Krakow, J., 301
Kramer, P. J., 219, 192, 193, 194, 197, 207, 364, 365
Krasilinikov, N. A., 406
Krasinskii, N. P., 404
Krasnovskii, A. A., 31, 402 403
Krastina, E. E., 421
Kratz, W. A., 52, 54, 55, 56, 57
Krebs, H. A., 13, 41, 338, 342
Kreger, D. R., 357
Kretovich, V. L., 222, 224, 408, 411
Krijthe, N., 387
Krippahl, G., 40
Kritskaia, L. M., 418
Kritzman, M. G., 410
Kriukova, N. N., 404, 406, 410, 412
Kroll, H., 184, 185, 186
Krotkov, G., 279, 288
Krueger, A. P., 129
Kruzhilin, A. S., 415, 421
Krylov, A. V., 419, 420
Kuff, E. L., 1
Kugler, I., 303, 305, 306, 316
Kuhn, R., 305
Kuiken, K. A., 173
Kunkel, L. O., 129, 130, 145, 146, 147, 154, 162
Kuprevich, V. F., 404, 406, 410
Kursanov, A. L., 401-36; 404, 405, 406, 407, 408, 409, 410, 411, 412, 413
Kurtz, E. B., 357
Kuse, G., 368
Küster, E., 116
Kuwada, G., 358
Kuykendall, J. R., 176, 184, 185, 186
Kuzin, A. M., 403, 404, 405, 406
Kuz'min, A. I., 418
Kuznetsova, M. S., 420
Kvamme, O. J., 309

L

Laborey, F., 305
Lacharme, J., 306
Lackey, C. F., 150
LaForge, F. B., 291
Lake, G. C., 216
Lambertz, P., 359
Lamer, V. K., 359
Lamey, H. A., 124
Lammerts, W. E., 192, 197
Landau, J. V., 106
Lane, R. H., 256
Lang, A., 203, 374, 375
Langford, N. H., 123
Langlet, O., 198
Lansdown, A. R., 309
Larsen, H., 53, 58, 60
Lasker, M., 292
Laties, G. G., 14
Latterell, R. L., 155, 156
Laurie, A., 198
Lavollay, J., 305
Lawrence, J. M., 317
Leach, J. G., 128
Leaper, J. M. F., 237
Lebedev, G. V., 412
Leblond, C. P., 96
LeClerg, E. L., 127
Leder, I. G., 272, 273
Ledingham, G. A., 129
Lee, S. B., 52
Leeper, G. W., 174
Lees, H., 58, 67
Leggett, J. E., 2, 3, 4, 9, 10, 11, 12, 16
Lehmann, E., 299
Lehninger, A. L., 284, 338
Leisle, F. F., 197
Leloir, L. F., 286, 287
Leman, V. M., 192, 193, 194, 196, 404
Lemnitskaia, V. K., 413
Leonard, C. D., 176, 178, 184, 187
Leonian, L. H., 131
Leopold, A. C., 205, 251, 257, 301, 308, 309, 366, 367, 368, 392
Le Page, G. A., 328
Leslie, I., 104
Lettré, H., 106
Levan, A., 108, 239
Levene, P. A., 291
Levitt, J., 13, 14
Lewin, R. A., 37
Lewis, C. H., 41
Lewis, F. J., 358
Lewis, M., 356, 359
Lewis, S. M., 53, 60
Lexander, K., 238, 249
Ley, J. de, 279
Leyon, H., 360
Lezandr, A. A., 192
Lhotský, S., 64
Libbert, E., 13, 249, 250
Liebenberg, A. R. von, 311, 316
Lieberman, M., 283
Liebich, H., 176
Liedtke, D., 86
Lightle, P. C., 135
Lilly, V. G., 123
Linder, P. J., 361
Lindner, R. C., 179, 181
Lindsay, W. L., 179, 180
Lindsey, A. J., 365
Lindstrom, E. S., 53, 59, 60, 62, 63
Lineweaver, H., 9, 56
Link, G. K. K., 268
Linkola, H., 128
Linkous, W. N., 232
Linskens, H. F., 7
Lipmann, F., 271, 291, 325, 326, 343
Lis, H., 226
Lisitsin, D. I., 409
Litvinov, L. S., 414
Liubimenko, V. N., 401, 403
Liverman, J., 82
Liverman, J. L., 200, 203, 204, 205, 313, 374, 387
Livingston, L. G., 159
Livingston, R., 31, 32, 45
Lobov, M. F., 415
Lockhart, J. A., 82
Logan, A. V., 356, 358, 359
Lohmann, J. M., see Meyer-Lohmann, J.
Lona, F., 305
Long, A. G., 309
Long, W. G., 13, 14
Loo, S. W., 135
Loomis, W. E., 282, 300, 355
Loughnane, J. B., 156
Loveless, A. R., 128
Lowe, E. P., 131
Lowenhaupt, B., 16
Löwenstein, A., 57
Lucas, E. H., 361
Luckwill, L. C., 312
Lugg, J. W. H., 215
Lull, R. S., 128
Lumry, R., 31, 34, 36, 41, 43 44
Lundegardh, H., 2, 4, 5, 13, 15, 16, 45, 342
Lundsgaard, E., 276
Lung Yuan, E., 40, 43
Lunt, O. R., 185
Luria, S. E., 97
Lutman, B. F., 122
Luttrell, E. S., 135
L'vov, S. D., 413
Lwoff, A., 92, 93
Lynch, R. S., 368
Lynch, V., 276, 286
Lysaght, A. M., 135
Lysenko, T. D., 420

M

Maaløe, O., 97
McAlister, E. D., 313, 377, 382, 395
McCalla, A. G., 215, 217, 220, 221, 223, 230
MacCarthy, H. R., 149
McClendon, J. H., 345
McCoy, E., 124
McCoy E. E., 116
McCready, R. M., 284, 286
Macdonald, I. R., 306
MacDougal, D. T., 128, 129,

135
Macdowall, F. D. H., 39
McElroy, W. D., 341
MacGee, J., 292
McGeorge, W. T., 181
McGeown, M., 291
McGonagle, M. P., 240
McGregor, S. M., 238, 240, 252
McIndoe, W. M., 95
McKay, R., 120, 131,
McKeen, W. E., 123
McKinney, H. H., 159
McLennan, E. I., 130
McMaster, R. D., 95, 104
McMurtrey, J. E., 66
McNair Scott, D. B., 279
McRae, D. H., 256, 257, 258
Magaldi, A., 294
Magee, W. E., 55, 57, 60, 61, 67
Magrou, J., 135
Mahler, H. R., 39, 61
Mai, W. F., 126
Maiboroda, S. S., 413
Mains, E. B., 117, 120, 133
Maksimov, G. A., 415
Maksimov, N. A., 401, 414, 415, 417
Maksimovich, A. E., 410
Malpress, F. H., 291
Mamul, Y. V., 403, 404
Mann, R. L., 229
Manning, W. H., 33
Manskaia, S. M., 412
Manten, A., 33
Mapson, L. W., 282, 293, 294 295, 317
Maramorosch, K., 129
March, R. B., 364
Margolina, K. P., 414, 415, 417
Markham, R., 148
Markley, E., 366
Marks, P. A., 289
Marmer, D. R., 250
Marshak, A., 95, 97, 104
Marshak, C., 95, 97, 104
Marsland, D., 101, 106
Marston, H. R., 173
Martell, A. E., 184
Marth, P. C., 364, 366
Martin, J. H., 302, 311
Martin, L. F., 159
Martius, C., 342, 345
Mashkovtzev, M. F., 412
Mashtakov, S. M., 412
Mason, M. M., 118
Massart, L., 3
Massee, G., 122, 123
Massini, P., 283
Matruchot, L., 116, 119, 120, 131
Matukhin, G. R., 416
Matveiev, M. I., 414
Matzke, E. B., 194
May, L. H., 288
Mayber, A. P., see Poljakoff-Mayber, A.
Mayer, A., 129
Mayer, A. M., 306, 308, 309
Mazalis, M., 13
Mazia, D., 103, 107
Mazurova, T. A., 410
Mechelke, F., 100
Mehler, A. H., 27, 28, 43, 44, 270, 281
Meischke, D., 314, 393
Melchers, G., 203, 375
Melhus, I. E., 122, 133, 135
Melik-Sarkisyan, S. S., 222, 224, 411
Melin, E., 126, 135
Mel'nikov, N. N., 420
Menge, J., 79
Mentzer, C., 306
Menzel, R. G., 11
Merenova, V. I., 405, 406
Mes, M. G., 79
Metcalf, M. M., 100, 101
Metcalf, R. L., 364
Metz, C. W., 102
Meyer, K. F., 129
Meyerhof, O., 29
Meyer-Lohmann, J., 84, 85
Michaelis, L., 42
Michel, B. E., 268
Michurin, I. V., 416
Mickelson, O., 120
Middendorf, F. G., 299, 300
Middleton, J. T., 155
Midge, G. H., 13
Mikhailova, E. S., 402
Milatz, J. M. W., 33
Millar, F. K., 16, 17
Miller, C. O., 13, 109
Miller, G. W., 14, 180
Millerd, A., 268, 269, 283, 338
Millikan, C. R., 183
Milner, H. W., 53
Milstrey, R. A., 123
Minina, A. K., 410
Minina, E. G., 405, 421
Mirksy, A. E., 95, 96, 103
Mishke, O. I. K., see Koblents-Mishke, O. I.
Mitchell, J. E., 155, 156
Mitchell, J. W., 361, 364, 365, 366
Mitsui, A., 347
Modess, O., 135
Moewus, F., 237, 254, 258, 305, 306, 307
Moewus, L., 254, 305
Mohr, H., 313
Mollart, D. L., 195, 211
Möller, A., 116, 135
Molliard, M., 116, 119, 120, 131, 135
Molotkovskii, G. Kh., 421
Montfort, C., 392
Moore, B., 53
Moore, S., 226
Morel, G., 117, 118, 367
Morgan, C., 241
Morgan, E. J., 317, 318
Mori, S., 234
Morimura, Y., 81
Morinaga, T., 311
Morita, S., 59
Morris, H. J., 54, 56, 57
Mortenson, L. E., 60
Mosbach, E. H., 293
Moshkov, A. S., 404
Moshkov, B. S., 192, 193, 194, 198, 209, 211, 382, 389, 421
Mountain, W. B., 118, 135
Mouros, M. A. de, 131
Mudretsova, K. A., 66
Mueller, G. C., 288
Mueller, L. E., 355
Mueller, R. T., 185
Muir, R. M., 257, 363
Müller, R., 254
Münch, E., 165, 195
Munch-Petersen, A., 288
Munnecke, D. E., 120
Murphy, P. A., 131
Murphy, W. H., 121
Murray, E. G. D., 122
Müssle, L., 86
Myers, J., 52, 54, 55, 56, 57

N

Nachmansohn, D., 1
Naismith, W. E. F., 227
Nakajima, H., 72
Nakamura, S., 313, 314
Nason, A., 341
Naves, Y. R., 305
Naylor, A. W., 283, 284, 300
Naylor, J., 110
Naylor, J. M., 109, 110
Neeb, O., 77, 81
Negelein, E., 315
Neger, F. W., 132
Neher, B. H., 84
Neiman, G. B., 413
Neiman, M. B., 413
Neish, A. C., 278, 285, 289
Nekrasova, T. V., 414, 422
Nelson, J. M., 286
Nelson, R., 157
Nestel, L., 14
Nétien, G., 258, 306, 308
Neumann, G., 301, 303, 313, 316
Newaschin, S., 128
Newburg, R. W., 347
Newcomb, E. H., 283, 300
Newcomer, E. H., 135
Newhall, A. G., 155
Newton, J. W., 53, 59, 60, 61, 62, 63
Newton, W., 119
Nezgovorova, L. A., 403, 421
Nichiporovich, A. A., 403, 421

Nickerson, W. J., 106
Nickerson, W. S., 107
Niedergang-Kamien, E., 368
Niederhauser, J. S., 127
Nielsen, N., 341
Nieva, F. S., see Sánchez Nieva, F.
Nigon, V., 95
Nikei, T., 105
Nikitin, I. N., 198
Nishigaki, S., 66
Noack, K., 176
Noble, R. J., 126
Noll, F., 99
Nordal, A., 288, 291
Nordlund, M., 286
Norris, L., 30, 62
Norris, L. T., 286
Norris, R. E., 30, 62
North, C. P., 186
Novick, A., 100
Novoa, S., 363
Nuernbergk, E. L., 79, 377
Nutile, G. E., 308, 315
Nyland, G., 155

O

Ochoa, O., 30
Ochoa, S., 30, 338, 339, 340, 346, 347
Ochs, G., 254
Ochs, R., 245, 254, 255
Odintzova, S. V., 65
Oesper, P., 29
Ogawara, K., 310
Ogur, M., 95, 334
Ohmura, T., 344, 345, 346, 347
Okajima, H., 183
Okanenko, A. S., 404, 407, 410
Okasako, Y., 313, 314
Oknina, E. Z., 416, 418
Okuda, A., 66
Oleinikov, T. V., 421
Olenicheva, L. S., 411
Olitsky, R. K., 129
Olmstead, C. E., 192, 194, 196
Olsen, C., 9, 181
Olsen, R. A., 368
Olsen, R. V., 184
Olsen, S. R., 180
Olson, A. A., 40
Olson, R. A., 257
O'Mara, J., 102
Ono, K., 310, 313
Oohusa, T., 317
Oota, Y., 318
Oparin, A. I., 409
Oppenheimer, H., 307
Ordin, L., 13, 15, 17, 177
Orgell, W. H., 356, 357, 358, 360, 361
Orillo, F. T., 135
Orth, H., 396
Orton, C. R., 135
Osborne, D. J., 363
Osborne, T. B., 215, 216, 217, 218, 221, 229, 230, 231
Oserkowsky, J., 177, 178, 367
Osipova, O. P., 403
Osnos, M., 272
Osterhout, W. J. V., 7, 8
Ostrovskaia, L. K., 413
Oswald, J. W., 155, 157
Ovcharov, K. E., 413, 414, 419, 420
Overbeek, J. Van, 355-72; 256, 358, 359, 360, 361, 362, 363, 368, 369
Overstreet, R., 3, 4, 12, 13
Owens, O. V. M., 385, 386

P

Pagan, V., 183
Page, H. J., 53
Palade, G. E., 360
Paladini, A. C., 286
Paleiev, A. M., 410
Palladin, V. I., 401
Panteleiev, A. N., 410
Pany, J., 325
Papi, F., 84, 85
Parija, B., 246
Pariser, R., 31
Parke, M. H., 363
Parker, M. W., 198, 202, 203, 313, 315, 374, 376, 377, 382, 383, 385, 386, 391, 394, 395, 397
Parker-Rhodes, A. F., 120
Parkinson, D., 7
Pauley, S. S., 191, 192, 197, 207, 209, 210
Pavlinova, O. A., 407, 409
Pavlov, A. N., 405, 411
Payette, A., 131
Payne, T. M. B., 7
Pedersen, K. O. 215
Peirce, G. J., 135, 147
Peive, Y. V., 406
Pelc, S. R., 95
Pelczar, M. J., 367
Pelletier, R. L., 120
Pereviazkina, L. M., 408
Perkins, H. F., 186
Perrault, C., 131
Perry, T. O., 191, 192, 197, 207, 210
Peterburgskii, A. V., 404
Petersen, A. M., see Munch-Petersen, A.
Peterson, B., 121
Pethybridge, G. A., 131
Petinov, N. A., 415
Petinov, N. S., 405, 411, 415
Petrie, A. H. K., 300
Petrochenko, E. I., 412
Petropavlovskaia, R. N., 421
Petrov, V. F., 419
Petrova, A. N., 409
Petrova, R. K., 404
Petrovskaia, T. G., 418
Pfister, C., 255
Phillips, J. E., 198
Pickholz, L., 311
Picklum, W. E., 299
Pilet, P. E., 238, 251, 255, 258, 259
Pinsky, M. J., 53, 60
Pintner, I. J., 56
Piper, S. H., 357
Piringer, A. A., 313, 384, 395, 397
Pirson, A., 2, 77, 78, 86, 325
Platt, J. R., 46
Plaut, G. W. E., 343
Plishevskaia, E. G., 405
Plowright, C. B., 129, 133
Pohl, R., 85, 244, 245, 250, 254, 255
Pol, B. van der, 80
Poljakoff-Mayber, A., 300, 309
Pollock, B. M., 109, 239, 242, 243, 247
Pontovich, V. E., 407
Poptsov, A. V., 308
Porret, D., 31
Porter, C. L., 124
Porter, G., 32
Porter, H. K., 288
Porter, J. W., 9
Porter, L. K., 179
Porter, R. H., 304
Portier, P., 135
Post, K., 198
Postgate, J. R., 67
Povolotskaia, K. L., 413
Powell, R. G., 363
Powers, J. A., 184, 185, 186
Prát, S., 120
Prescott, D. M., 103
Preston, C., 180
Preston, R. D., 365
Preston, W. H., 366
Prianishnikov, D. N., 401, 404
Price, L., 375, 376, 385, 386
Price, N. O., 232
Price, W. C., 128
Priestley, J. H., 195
Pringsheim, E. G., 54
Pringsheim, P., 34
Proctor, B. E., 79
Prokhorova, P. A., 408
Prokof'ev, A. A., 410, 412, 413
Prokoshev, S. M., 410, 412, 413
Protaschik, N. E., 406
Provasoli, L., 56
Pulatova, M. P., 415
Punnett, T. R., 43
Pushkareva, M. I., 404
Pushkinskaia, O. I., 413
Putman, E. W., 288

Q

Quayle, J. R., 290

Quensel, O., 216, 217
Quinn, E. M., 288

R

Rabideau, G. S., 380, 388, 389
Rabinowitch, E., 31
Rabinowitch, E. I., 25, 26, 31, 34, 35, 44, 325, 328, 342
Rabinowitz, J. L., 285
Racker, E., 270, 272, 273, 290, 291, 339
Racker, F., 29
Radu, V., 300
Rakitin, I. V., 413, 418, 419, 420
Raleigh, G. J., 308
Raminsky, I. R., 410
Ramirez-Silva, F. J., 182, 183
Randolph, L. F., 306
Ransom, S. L., 246
Raper, K. B., 130
Raphael, J. C., 135
Rappleye, L., 198
Rasmussen, L. W., 302
Ratner, E. I., 404, 405, 406, 410, 416
Rau, W., 80
Rauen, H., 270
Rawlins, T. E., 120, 144 356, 357
Ray, J., 134
Razumov, V. I., 421
Ready, D., 237
Rector, C. W., 46
Reddick, D., 155, 157
Rediske, J. H., 3
Reed, G. B., 279, 288
Reed, H. S., 134, 305
Reeve, W., 366
Reichenberg, D., 4
Reid, M. R., 54, 55
Reinders-Gouwentak, C. A., 391
Reinert, J., 251
Reinhold, L., 360
Reith, W. S., 238, 241
Reuther, W., 176, 178, 183
Reznichenko, M.S., 222
Rhoades, M. M., 93
Riazantsev, A. V., 417, 418
Rice, E. L., 366
Rice, L., 292
Rice, M. A., 124, 126
Rich, C. E., 217, 220
Rich, S., 363
Rideal, E. K., 227, 361
Rieger, C., 60
Riley, V., 14
Ripley, G. W., 365
Ris, H., 94, 98, 103, 104
Robbins, W. J., 122
Robbins, W. R., 14, 182, 183, 186
Robbins, W. W., 303
Roberts, D. A., 163, 164, 165
Roberts, E. A., 79
Roberts, E. H., 255
Roberts, E. O., 135
Roberts, F. M., 163
Roberts, I. Z., 5
Roberts, R. B., 5, 6, 7
Robertson, R. N., 6, 13, 14
Robertson-Cuninghame, R. C. 361
Robinson, E., 238, 241, 309
Robinson, W., 176, 178, 181
Rodionov, A. P., 418
Roe, E. I., 191
Roelofsen, P. A., 355, 357, 359
Rohrbaugh, L. M., 366
Romanova, I. V., 420
Rombach, J., 330, 335
Rodenburg, J. W. M., 374, 377, 390, 391
Ropp, R. S., de, 366
Rosen, G. U., 95, 100
Rosenberg, J. L., 334, 337,
Rosenberg, L., 40
Rosenberg, L. L., 29, 44, 269, 340, 342, 343, 344, 345, 349, 351
Rosenberg, R. E., 406
Rosenberg, T., 9
Rosenheiser, H. A., 125
Rosenstock, G., 392
Rossman, E. C., 361
Rothblühr, L., 37
Rothschild, I., 84
Rothstein, A., 11
Rouatt, J. W., 7
Rousseau, J., 307
Rozenfeld, E. L., 409
Rozhkov, M. I., 413
Rozhkova, N. K., 420
Ruben, S., 53, 325, 326, 327, 329, 340, 350, 351
Rubin, B. A., 408, 409
Rue, J. L., 160
Rueckert, P. R., 288
Rufelt, H., 258
Ruge, A., 86
Ruge, U., 389, 390
Russell, R. S., 3, 10, 19
Ryzhov, S. N., 415

S

Sabinin, D. A., 401, 405
Sachs, J., 310, 373
Sachs, R. M., 197
St. John, J. C., 232
Salmon, E. S., 155
Salomko, E. A., 418
Saltman, P. D., 269
Saltykovski, M. I., 417
Samish, R. M., 192, 206
Samoilova, S. A., 405, 406
Samokhvalov, G. K., 404
Samuel, G., 129, 154, 162
Samygin, G. A., 192, 417
Sanadi, D. R., 284
San Antonio, J. P., 238, 239, 247
Sánchez Nieva, F., 361
Sandeen, M. I., 84, 85
Sandegren, E., 215
Sander, G., 110
Sandstrom, B., 238
Sanikidye, A. C., 178
Santen, M., 27
Sappin-Trouffy, 134
Saprygina, E. S., 417
Sarkisyan, S. S., see Melik-Sarkisyan, S. S.
Sasa, T., 81
Satarova, N. A., 418, 419
Säverborn, S., 218
Savile, D. B. O., 123, 134
Savulescu, A. A., see Aronescu-Savulescu, A.
Sawyer, C. N., 66
Sax, K. B., 95
Sayed, G. S., 117
Sceglova, O. A., 197
Scepotjev, F. L., 198
Schader, E., 305, 306, 307
Schaede, R., 64
Schatz, A., 334
Scheer, C. van der, 377, 379, 387, 395
Schenck, H., 99
Schimper, A. F. W., 99
Schlegel, H. G., 39
Schmidle, A., 74
Schmidt, E. W., 118
Schmitz, J., 79
Schneider, E., 31
Schön, J., 77, 78
Schön, W. J., 77, 80
Schopbach, R., 56
Schopfer, W. H., 240, 248
Schraeder, F., 101
Schreiber, F., 127
Schreiner, O., 305
Schröder, W., 40
Schulz, G. A., 194, 209, 210
Schwabe, W. W., 84
Schweizer, G. 116
Schwemmle, B., 84
Schwendiman, A., 310
Schwinck, L., 334
Scott, D. B. M., see McNair Scott, D. B.
Scott, F. M., 355, 356, 359, 360, 365
Scott, G. D., 64, 65
Scott, G. T., 8, 11
Scully, N. J., 383
Sedenko, D. A., 406
Seeberg, V. P., 186
Seegmiller, C. G., 284, 288
Segal, H. L., 29
Segueira, L., 367
Seifriz, W., 72
Seifter, E., 283
Selders, A. A., 3
Sell, H. M., 361

Sergeiev, L. I., 416
Servin, L., 127
Sevastianov, V. I., 416
Sevenster, P., 367
Shakhov, A. A., 416
Shands, H. L., 310
Shanes, A. M., 2, 5
Shannon, L. M., 185
Shanor, L., 135
Shantarovich, N. S., 413
Shantz, E. M., 110, 111
Sharapov, N. I., 410
Sharashidze, N. M., 418
Shardakov, V. S., 414, 415
Sharp, E. L., 125
Shavlovskii, G. M., 406
Shaw, M., 120
Shaw, W. C., 308
Shcheglova, V. F., 419
Shear, C. L., 116
Sheffield, F. M. L., 143, 150, 159
Sherman, F. G., 3
Sherman, W. C., 173
Shettles, L. B. 272
Shibata, K., 40, 43, 44, 81
Shimogawara, G., 317
Shipton, M. E., 239, 256, 258, 259, 260
Shive, J. W., 178
Shkol'nik, M. Y., 406
Shkvarnikov, P. K., 418, 419
Shlyk, A. A., 403
Shmuk, A. A., 405, 407, 412
Shollenberger, J. H., 232
Shooter, E. M., 227, 228
Short, W. A., 54, 55
Shrift, A., 11
Shu, P., 129
Shubina, A. I., 409
Shuck, A. L., 306
Shug, A. L., 61
Shultze, M. O., 173
Shumakova, R. R., 417
Shutov, D. A., 418
Shvedskaia, Z. M., 421
Sibatani, A., 96
Siegel, J. M., 58, 59
Siegel, S. M., 367
Silva, F. J. R., see Ramirez-Silva, F. J.
Simonis, W., 331, 333, 334
Singh, R. N., 65
Sirotenko, A. A., 412
Sisakyan, N. M., 402, 403, 409, 414
Sisterm, W. R., 47
Sitnikova, O. A., 418, 419
Sjöstrand, F. S., 360
Skazkin, F. D., 414
Skinner, J. J., 305
Skipper, H. E., 54, 55
Skoog, F., 54, 56, 109, 110, 256, 257, 261, 364, 367, 368
Skorobogatova, E. P., 413
Skoss, J. D., 357, 359, 364
Slater, C. E., 350
Slater, E. J., 338
Sluysmans, C. M. J., 377, 378, 391, 395, 397
Smeets, L., 391
Smellie, R. M. S., 95, 96
Smirnov, A. I., 405, 407, 412
Smirnov, E. N., 413
Smirnova, T. I., 411
Smith, E. B. S., 288
Smith, F. G., 125, 283, 300
Smith, G. M., 85, 98, 99, 101
Smith, J. H. C., 44, 46
Smith, K. M., 121, 123, 148, 150
Smith, L., 40, 45
Smith, P. F., 176, 178, 182, 183
Smith, R. E., 131
Smyrniotis, P. Z., 272, 273, 288, 289, 290
Snieszko, S. F., 131
Snyder, W. C., 120, 134, 155
Sobolevskaia, O. I., 413
Söding, H., 195
Sokolov, A. V., 406
Sokolova, V. E., 407, 408
Soldatenkov, S. V., 410
Sollberger, A., 80
Sollner, K., 4
Solovieva, M. A., 418
Somers, I. J., 178
Sparrow, A. H., 95, 100, 120
Spaulding, P., 134
Spear, I., 79
Spears, J. F., 126
Specht, A. W., 19, 176, 178, 181, 182, 183
Speirs, M., 172
Speirs, R. S., 84
Spikes, J. D., 31, 34, 36, 41, 43, 44
Spivakovski, N. D., 418
Spoehr, H. A., 53, 375, 376
Sprinson, D. M., 289
Spruit, C. J. P., 37, 40, 43
Srinivasan, P. R., 289
Stafford, H. A., 270, 272, 283, 294
Staiger, H., 100, 108
Stakman, E. C., 123, 124, 127, 128
Stalfelt, M. G., 73
Stamforth, D. W., 361
Stanton, T. R., 302, 311
Starr, L., 313, 387
Stauffer, E., 103
Stauffer, J. F., 244, 327, 328, 329, 330, 342
Stebler, F. G., 311
Steere, R. L., 158
Steeves, T. A., 367
Stefanovskii, I. A., 414
Stein, G., 315
Stein, W. H., 226
Steinbach, H. B., 11
Steinberg, R. A., 118
Stepanenko, B. N., 409
Stepanova, E. M., 143
Stepanovich, K. M., 222, 224, 411
Stepka, W., 238
Stern, H., 91-114; 103, 104, 106
Stern, K. G., 172
Stetten, D. Jr., 280, 282
Stevens, C. E., 96
Stevens, N. E., 127
Stevens, P. G., 5, 6
Stevens, R. B., 127
Steward, F. C., 2, 16, 17, 110, 111, 180, 215
Steward, R. N., 123
Stewart, I., 176, 178, 184, 187
Stewart, V. B., 155
Stich, H., 101, 104, 106, 336
Stiles, W., 299
Stiller, M. L., 283
Stock, F., 118, 133
Stock, J. van der, 3
Stokes, J. L., 65
Stokes, P., 301, 312
Stolwijk, J. A. J., 373-400; 377, 378, 379, 380, 381, 382, 383, 384, 385, 388, 389, 390, 391, 393, 394, 395, 397
Stoppani, A. O. M., 30, 62
Storey, H. H., 148, 161
Stout, M., 306
Stout, P. R., 4
Stowe, B. B., 249
Straib, W., 134
Strasburger, E., 99
Street, H. E., 240, 252, 255, 258, 261
Strehler, B. L., 39, 41, 47, 334, 335, 336, 349, 350, 351
Strelnikova, M. M., 413
Strepkov, S. M., 409
Strogonov, B. P., 416
Strong, F. M., 109
Stumpf, P. K., 13, 268, 269, 273, 325
Stutz, R. E., 283
Sudzuki, F., 363
Sulaiman, M., 65, 66
Sulakadze, T. S., 417
Sunderland, N., 309
Suneson, C. A., 155, 157
Sunner, S., 42
Sushkevicn, T. I., 410
Sushkova, A. S., 410
Sussex, I. M., 240, 252
Sussman, A. S., 301
Sutcliffe, J. F., 3, 4, 8, 239, 241, 246
Sutter, E., 13
Suzuki, K., 59
Svedberg, T., 215, 218
Sveshnikova, I. N., 410
Swann, M. M., 105
Swanson, C. A., 365
Swanson, C. P., 237
Swanson, C. R., 308
Swenson, S. P., 232
Swick, R. W., 96

Swift, H., 95
Switzer, C. M., 283, 300
Sylven, H., 191, 192, 193, 210
Sylvester, E. S., 149
Syverton, J. T., 121

T

Tabentskii, A. A., 403
Tager, J. M., 283, 300
Tahara, D. M., 96
Takahashi, W. N., 129, 356, 357
Takashima, S., 59, 347
Tamiya, H., 36, 81
Tanada, T., 12
Tanner, K. G., 129
Tarantola, M., 318
Tatum, E. L., 123
Taves, C., 238, 247
Taylor, J. H., 95, 104
Tchen, T. T., 30
Teiss, R. V., 403
Tenebaum, E., 120
Terenin, A. N., 402
Terent'ev, V. M., 406
Terent'eva, M. V., 403
Tewfik, S., 269
Therman, E., 94, 99, 108
Thimann, K. V., 79, 234, 244, 249, 250, 251, 252, 254, 307, 309, 366, 367
Thoday (Sikes), M. G., 147
Thomas, J. B., 33, 44
Thomas, W. D., Jr., 145
Thomas, W. D. E., 364, 366
Thompson, J. F., 215
Thompson, J. L. C., see Cloudsley-Thompson J. L.
Thompson, R. C., 308
Thomson, B. F., 108
Thornberry, H. H., 144
Thorne, D. W., 176, 178, 179, 180, 181, 182, 183
Thorne, G. N., 364
Thorne, J. A., 357, 359, 364
Thornton, H. G., 122
Thresh, R., 239, 254
Tietz, N., 309
Tillaev, R. S., 410
Tilly, F., 313
Timiriazev, K. A., 401
Timonen, S., 99, 106
Tiselius, A., 215
Tjia, J. E., 330, 332, 337
Todd, A. R., 309
Todd, G. W., 395
Tolbert, N. E., 276, 277, 278
Tolmach, L. J., 338
Tolman, B., 306
Tombesi, L., 318
Tompkins, C. M., 144
Tonhazy, N. E., 367
Tonzig, S., 318
Toole, E. H., 299-324; 84, 202, 203, 204, 299, 300, 302, 307, 310, 311, 313, 314, 315, 316, 374, 382, 383, 385, 386, 397
Toole, V. K., 299-324; 84, 202, 203, 204, 302, 307, 310, 311, 313, 314, 315, 316, 374, 382, 383, 385, 386, 397
Torrey, J. G., 237-66; 241, 243
Totter, J. R., 334
Touster, O., 292
Tove, S. R., 53, 60
Traversi, B. A., 155
Treherne, J. E., 363
Trelease, S. F., 11
Treumann, W. B., 309
Trezzi, F., 318
Tribukait, B., 84
Tronchet, A., 72
Tronchet, J., 72
Trumpf, C., 375
Tsukervanin, I. P., 420
Tsvetkova, I. V., 414, 415
Tucker, C. M., 131
Tuieva, O. F., 405, 407, 411
Tukey, H. B., 355
Tulasne, C., 116
Tulasne, L. R., 116
Tumanov, I. I., 405, 407, 417, 418
Turchin, F. V., 405
Turetskaia, R. Kh., 419, 420
Turkina, M. V., 407, 408
Turner, J. S., 276
Turner, W. F., 150, 152, 154, 159, 160
Tuttle, L. C., 291
Tyler, G. J., 309

U

Uchevatkin, F., 405
Udalova, L. A., 404
Uebelmesser, E. R., 74
Ukhina, S. F., 405, 406, 407, 411
Umbreit, W. W., 327, 328, 329, 330, 342
Uppal, B. N., 158
Uritani, I., 283
Usmanov, Kh. U., 410
Uspenskaia, Zh. V., 411

V

Vaartaja, O., 192, 210
Vallance, K. B., 312
Valleau, W. D., 144, 155, 156
van den Honert, T. H., see Honert, T. H., van den
van der Hart, J. M., see Hart, J. M. van der
van der Pol, B., see Pol, B. van der
van der Scheer, C., see Scheer, C. van der
van der Stock, J., see Stock, J. van der
Van der Veen, R., 192, 196, 391
van Genderen, H.,see Genderen, H. van
Van Niel, C. B., 30, 34, 35, 51, 58, 59, 60, 62, 63
van Overbeek, J., see Overbeek, J. van
Varzandeh, M., 144
Vasil'ev, I. M., 414
Vasil'eva, N. A., 409
Vasil'eva, N. G., 414, 415
Vatter, A. E., 44
Vegis, A., 191, 203
Veldsta, H., 363
Venezian, M. E., 318
Vennesland, B., 29, 30, 270, 272, 294, 317, 318,
Venter, J., 77
Vercier, P., 306
Vercruysse, R., 279
Vereshchagin, A. G., 405, 407, 411
Vergnano, O., 182, 183
Verkhovtseva, T. P., 409
Vermund, H., 96
Vernon, L. P., 40, 44, 58, 365
Vervelde, G. J., 4, 7
Vickery, H. B., 216
Vieregg, A., 240, 252, 254
Viets, F. G., 4, 12
Vinet, J. G., see Guérillot-Vinet, J.
Vinogradov, A. P., 403
Vinokur, R. L., 405, 407
Virgin, H. I., 46, 73, 359, 393, 396
Virtanen, A. I., 51, 128, 286
Vishniac, W., 27, 28, 29, 38, 290, 325, 338, 339, 340, 346, 347
Visscher, M. B., 84
Vittorio, P. V., 288
Vladimirov, A. V., 404
Vlasiuk, P. A., 406
Vogler, K. G., 328, 330
Vogt, I., 249
Voinovskaia, K. K., 402, 403
Volkers, W. S., 19
Volkoslavskaia, E. N., 421
Volubaieva, N. P., 408
von Euler, H., see Euler, H. von
von Guttenberg, H., see Guttenberg, H. von
von Karschon, R., see Karschon, R. von
von Liebenberg, A. R., see Liebenberg, A. R. von
Von Saltza, M. H., 109
Vorab'eva, M. N., 412
Voskresenskaya, N. P., 389, 403, 421
Voss, J. J., 35
Vouk, V., 57, 66
Vyskrebentseva, E. I., 404, 407, 410, 412
Vyval'ko I. G., 413

W

Wadleigh, C. H., 179, 180
Wagenknecht, A. C., 60,61, 252
Wagner-Jauregg, T., 270
Wahlin, H. B., 52, 53
Wainio, W. W., 14
Walker, B. E., 99
Walker, D. A., 283
Walker, D. R., 360, 364
Walker, J. C., 119
Wall, J. S., 60, 61
Wall, R. F., 179
Wallace, A., 178, 185, 186
Wallace, T., 176, 181
Wallihan, E. F., 176
Wallin, I. E., 135
Walp, L., 56
Walter, H., 2
Wangermann, E., 85
Wann, F. B., 53, 176, 178, 181, 182, 183
Wanner, H., 238, 246
Warburg, O., 40, 269, 315, 334
Ware, W. M., 155
Wareing, P. F., 191-214; 84, 191, 192, 193, 194, 195, 196, 197, 198, 199, 201, 202, 205, 206, 208, 209, 317
Wareing, V., 82, 84
Waring, W. S., 177
Warner, R. C., 227
Wassink, E. C., 373-400; 33, 330, 332, 335, 337, 373, 377, 378, 379, 380, 381, 383, 387, 391, 393, 395, 397
Watanabe, A., 57, 65, 66
Watanabe, F., 180
Watson, D. P., 304
Watson, J. D., 96, 97
Watson, M., 152
Watson, M. A., 149
Watson, W. E., 31
Waygood, E. R., 172
Weaver, R. J., 358, 363
Webb, H. M., 84, 85
Webb, M., 104
Webster, G. C., 172
Webster, T. A., 53
Weeks, D. C., 13
Weinberger, J. H., 150, 152, 154, 159
Weinstein, L. H., 14, 182, 183, 186
Weintraub, M., 128
Weintraub, R. L., 357, 359, 364, 385, 386
Weisbach, A., 290
Weiss, J., 31
Weiss, M. G., 178, 182
Weissman, G. S., 11
Wells, D. G., 232
Wells, H. G., 216
Went, F. W., 84, 302, 310, 366, 367, 368, 385, 386
Werkman, C. H., 177
Wessels, J. S. C., 39, 43, 44 343, 350
Westerdijk, J., 116, 117, 130
Westergaard, M., 302
Westerheim, von W. W., see
Wettstein-Westerheim, von W.
Westheimer, F. H., 43
Westlake, D. F., 58
Wetmore, R. H., 367
Wetter, R. L., 221, 233
Wettstein-Westerheim, von W., 211
Whatley, F. R., 29, 37, 39, 44, 177, 269, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 349, 351, 352
Wheeler, H. E., 122
Whiffen, A. J., 118
White, M. J. D., 94, 100
White, P. R., 117, 119, 121, 240
White, R., 366
Whitley, E., 53
Whitney, J. B., Jr., 365
Whittam, R., 5
Whittingham, C. P., 15
Wiebe, G. A., 302, 311
Wiebe, H. H., 19, 364, 365
Wigglesworth, V. B. 110
Wight, W., 195
Wightman, F., 240, 241
Wilbrandt, W., 9
Wilde, M. H., 238
Wildman, S. G., 215
Wilhelm, P., 133
Wilken, W., 186
Wilkins, M. J., 13, 14
Wilkinson, J., 365
Williams, A. E., 52, 66
Williams, R. F., 2
Williamson, D. H., 291
Willis, L. G., 176
Wilson, A. R., 128
Wilson, A. T., 26, 27, 28, 286, 290, 347
Wilson, P. W., 51, 52, 53, 54, 59, 60, 61, 63
Wilson, T. H., 41
Wilson, W. L., 110
Wingard, S. A., 128
Winter, G., 52, 54, 62, 64
Wintermans, J. F. G. M., 330, 331, 332, 336, 337, 348
Withrow, R. B., 313, 375, 376, 386, 390,
Witt, H. T., 45
Wohl, K., 7
Wolf, E. A., 122, 135
Wolf, F. T., 122, 135
Wolfe, M., 51, 52, 54, 55, 56, 60, 61
Wolken, J. J., 360
Wood, F. C., 108
Wood, W. A., 292
Woodbridge, C. G., 181
Woronin, M., 128
Wright, B. E., 63
Wurgler, W., 258
Wylie, R. B., 358
Wynd, F. L., 127
Wyssling, A. F., see Frey-Wyssling, A.

Y

Yamada, E., 313, 314
Yamaguchi, M., 66
Yamamoto, Y., 318
Yardeni, D., 306
Yarwood, C., 119
Yarwood, C. E., 115-142; 118, 120, 121, 123, 125, 126, 127, 128, 129, 131, 132, 158
Yeatman, J. N., 359, 364
Yin, H. C., 367
Yocum, C. S., 40
Young, D. W., 366
Young, H. E., 133
Young, R. E., 284
Young, V. K., 33
Yuan, E. L., see Lung Yuan, E.

Z

Zabluda, G. V., 414
Zain ul Abidin, 84
Zaitseva, N. I., 413
Zalenskii, O. V., 403, 404, 408
Zaprometov, M. N., 406, 412, 413
Zdanova, L., 205
Zeevaart, J. A. D., 381, 384, 385, 394
Zemit, V. E., 306
Zeuthen, E., 95
Zhdanova, L. P., 410, 412
Zholkevich, V. N., 408, 415, 417
Zhurbitskii, Z. O., 408
Zikhman, O. K. K., see Kedrov-Zikhman, O. K.
Zill, L. P., 276, 277, 278
Zimmerman, A., 117
Zimmerman, A. M , 106
Zimmerman, K., 304
Zobell, C. E., 67
Zöllner, N., 344
Zöttl, H., 240

SUBJECT INDEX

A

Abscission, 192, 194
Absorption, of ions, 2-7
Acetaldehyde, 268
Acid synthesis, 404
Adenosinetriphosphate,
  in photosynthesis, 39, 42, 325-52
After-ripening, 312
Ageing, 193
Agriculture, see 401-36
Alanine, 277, 278, 403, 406
  formation of, 389
Albumin, 220, 221, 223, 233, 411
  enzymes in, 224
Algae
  "carriers" in, 7
  isotype exchange in, 8
  light and, 81
  "water space" in, 6
  see also Blue-green algae
Alkaloids, 412
Amino acid, 7, 216, 217, 220, 223, 226, 232-34
  formation of, 389, 403
  in germination, 312
  and photosynthetic bacteria, 59
  in root elongation, 241
Amitosis, 99
Amphoteric systems, 4
Anions
  absorption and, 14-15
  iron chlorosis and, 185
Antiauxins, and root elongation, 256-62
Antibiotics, 4
Antimetabolites, in root elongation, 248-49
"Apparent free space", 5-6
Appressoria, 125
Arachin, 227-30
Ascorbic acid
  formation, 293-95
  germination and, 317-18
  mitosis and, 106
Ascorbic acid oxidase, and iron chlorosis, 175
Athiorhodaceae, nitrogen fixation in, 58-60
Auxin
  absorption, 360
  action and red light, 389
  carbohydrate effect on, 365-366
  cell division and, 109-10
  distribution pattern of, 364-65
  endogenous rhythm and, 72, 79-80, 85
  germination and, 306
  molecular structure and, 362-63, 366
  penetration of, 363-64
  pH effect, 360-61
  photoperiodism and, 195, 205
  polar transport of, 366-68
  rate of movement of, 365
  ripening and, 419
  root elongation and, 238, 239, 243-45, 247, 249-62
  temperature and, 361-62
  translocation of, 364-66
  water uptake and, 17
  wetting agents and, 361
Axenic culture, see Obligate parasitism

B

Bacteria
  as obligate parasites, 129
  photosynthesis in, 30-34
  photosynthetic growth of, 58-61
  photosynthetic nitrogen fixation in, 52-53, 58-61
Bacteriochlorophyll, 31-34
Barley, seed globulins of, 217-220
Bicarbonate ion, and iron chlorosis, 178-80
Biochemistry of mineral nutrition, 12-17
Bleeding, 77
Blue-green algae
  ecological role, 65-67
  growth of, 54-58
  nitrogen fixation in, 51-52, 54-58
  symbioses of, 63-65
Blue light, see Light quality
Buds
  cell division in, 108-9
  dormancy in, 85, 192, 195-203, 208
  light quality and, 378
Bundle sheath, 358

C

Calcium
  and iron chlorosis, 180-81
  transport, 16
Cambial activity, photoperiodism and, 194-95
Cambium, virus infection of, 150
Carbohydrate
  appearance in germination, 301
  breakdown in plants, see Carbohydrate breakdown
  effect on auxin uptake, 365-366
  formation and light quality 404
  as protective colloid, 223
  transfer by dodder to host, 146
  transport and virus invasion, 161-65
Carbohydrate breakdown, 287-95
  Embden-Meyerhof-Parnas pathway, 267-69
  pentose phosphate pathway, 269-83
  pyruvate consumption, 283-84
  transformations, misc., 284-95
Carbon dioxide
  assimilation of, 325-29
  endogenous rhythm and, 79
  fixation of, 289-90, 325-29
  and germination, 303
  and iron chlorosis, 179
  and phosphorylation, 337, 343-44
  and polyphosphates, 336
  reactions in photosynthesis, 26-30, 39
  reduction of, 27-30
  see also Carbohydrate breakdown
Carbon monoxide, as inhibitor, 13
Carbonic acid, 404
Carotenoids in photosynthesis, 33
Carriers in ion transport, 7-12, 16-18
Catalase, 173, 175, 177
  iron salts as, 172
Cations
  absorption and, 14-15
  exchange, 2-5
Cell
  changes in root elongation, 238-44, 246, 253, 261
  division, see Cell division
  elongation of in germination, 299-300, 306, 307
  ion exchange in, 6-7
  iron in, 176
  pigment energy transfer in,

32-34
Cell division
cell type and, 107-9
cytoplasmic components, 92-93
intercellular relations, 107-11
intracellular relations, 97-99
mitosis, 99-101
organic environment and, 109-11
physiology of, 91-111
Cellulose manufacture, 246, 278, 285, 410
Chelates, 184
Chemosynthesis, 328
Chlorellin, 396
Chlorobacteriaceae, nitrogen fixation in, 58-60
Chlorophyll
and obligate parasites, 117
photochemical reactions of, 31-32, 35-37
photoreduction in, 402-3
protein and, 403
synthesis of, 403
triplet state of, 32, 35
virus infection and, 150
see also Photosynthesis
Chloroplast
carbon dioxide reduction by, 29-30
enzymes, 403, 409
iron and, 176-77
light and, 37-40
multiplication of, 93
phosphorylation in, 337-52
phototactic movement of, 403
reactions, 43-44
Chlorosis, iron, see Iron chlorosis
Chromosomes
multiplication of, 93-98
see also Mitosis
Chytrids, see Obligate parasitism
Climate effect on plant proteins, 232-33
Cold resistance, 401
Coleorhiza, 299, 300
Conarachin, 227-28
Copper, iron chlorosis and, 173-75, 178, 181, 183
Coumarin, 247
and germination, 306, 315, 318
"Critical day-length", 375
Cuticle
chemical composition of, 357
penetration of, 358-59
structure of, 355-56
surface chemistry of, 357-58
Cutin, 356, 357
Curly top, 145, 146, 158, 162
Cytochrome system, 80, 342
iron and, 177
Cytochromes, 172
and germination, 315
ions and, 13
oxidase translocation and, 407
in photosynthesis, 37-40, 42, 43
of photosynthetic bacteria, 58
Cytoplasm
cell division and, 98, 100-1
ions in, 7
multiplication in, 92-93
mutation in, 93
virus infection and, 159

D

Desert
flora, 302
see also Drought resistance
Diffusion, ionic, 5-7
Dismutation reactions, 25
Diurnal endogenous rhythms, 73-85
in animals, 84-85
deviations, 76
examples, 73-76
metabolic basis, 77-81
photoperiodism and, 81-84
regulating factors, 73-76
temperature and 76-77
thermoperiodism and, 84
tidal rhythms, 85
Dodder
as experimental transfer agent, 146
as virus transmitter, 146-48
Dormancy, 85-86
breaking of, 195-97
and germination, 301, 305, 312
induction of, 192-94
nuclei and, 418
photoperiodic perception and, 198-200, 205
recent work in U.S.S.R., 418-19
Downy mildew, see Obligate parasitism
Drought resistance, recent work in U.S.S.R., 414-18
Dyes, 4

E

Ecology
nitrogen fixation and, 65-67
photoperiodism and, 207-11
Embden-Meyerhof-Parnas pathway, 267-69, 275, 280, 282
Embryo, viruses and, 155-57
Endogenous rhythms, 71-87
annual, 85-86
definition, 71
diurnal, see Diurnal endogenous rhythms
short periods, 71-73
Endosperm, germination and, 302, 303, 304, 306
"Energid" theory, 98
Energy
endogenous rhythm and, 80
provision in mitosis, 104-7
transformations in photosynthesis, 25-47
carbon dioxide fixation and, 26-27
pigments and, 32-34
requirements, 41-43
Enzymes
in CO_2 fixation, 27, 29, 289
chloroplast, 403
in EMP pathway, 268
in dehydrogenation, 269
digestive, 356
endogenous rhythm and, 77, 79, 86
in germination, 300, 308-9
and glucose assimilation, 333
in glycolysis, 27
iron and, 172, 175, 179
legumin as, 224
in mineral transport, 9
mitochondrial, 14
in pentose phosphate pathway, 27, 269-74, 279-80
and phosphorylation, 337-40, 343
protein as, 216
of root, 406
and root elongation, 241, 243
salinity and, 416
in sucrose synthesis, 286
synthesis, recent work in U.S.S.R., 408-14
vicilin as, 224
Epidermis, 8
absorption and, 356, 358, 364
cell changes in root elongation, 238
as virus shield, 143-44
Erysiphaceae, as obligate parasite, 132-33
Erythulose phosphate, 288
Ethanol, 268
Etiolation, light quality and, 374, 375, 379, 385-88
Exchange, 4, 8

F

Fat synthesis, 410
Fermentation, 401
Ferritin, 173
Flagellar movement, 71

Flowering
light quality and, 377-81, 383-84
photoperiodism and, 197-98, 200, 206, 208
virus infection and, 157
Fluorescence, 32, 34, 46-47
Foliar nutrition, 405
Food transport and virus movement, 163-65
Freezing, plant resistance to, 417, 418
Fungi
in culture, 134-35
see also Obligate parasites

G

Gametes, 85
Gas
exchange measurement, 43-44
exchange in germination, 302-3
Gene for iron utilization, 178
Genetics, photoperiodic response and, 209-10
Germination, 299-319
ascorbic acid and, 317-18
coat effects, 302-5
glutathione and, 317-318
inhibitors and promotors, 305-10
light and dark and, 312-16
light quality and, 374, 382-383
organic acids and, 410
photoperiodism and, 202-4, 316-17
plant proteins and, 233-34
temperature and, 202-4, 310-12
Globulins, 215-20
as dissociating systems, 227-30
of seeds, 215-26, 230-33
Glucose
assimilation, 332-33
breakdown, 267, 268, 276, 278-80, 282, 284, 286, 292, 293
as mitotic stimulant, 110
Glucose-6-phosphate dehydrogenase, 270-72
Glutathione, 270, 272
germination and, 317-18
Gluten, 217
Glycinin, 229
Glycolysis, inhibition of, 13
Graft transmission of viruses, 144-46
Growth
dry weight and light quality, 380
extension, photoperiodism and, 192-94, 207-9
"flushes", 193-94
inhibition of, 419-20
ion absorption and, 16-17
light quality and, 373-97
nutational movement and, 72
physiology of, 420-22
recent work in U.S.S.R., 419-22
stimulation of, 419-20
see also Diurnal endogenous rhythms
Guttation, 77
Gymnosperms, cell division in, 98

H

Haustoria, 124
of dodder, 146
as virus transmitter, 147
Herbicides, 419-20
Heteroecism, 128
Hexokinase, 2, 332, 347
Hill reaction, 43, 239
Hormone
root elongation and, 261
see also Auxin
Horticulture, plant irradiation, 389-92
Hydrogen
photoproduction in bacteria, 58-59
production in photosynthesis, 37
Hydroxylamine, in germination, 310

I

Immunology protein tests, 216
Infrared light, see Light quality
Inhibition
of auxin by riboflavin, 394
dehydrogenase and, 402
of growth, 419-20
of growth by light quality, 381, 384
of mesocotyl, 385, 386
of mitosis, 100, 105
mitotic control and, 109-10
mitotic stimulation, 110-11
of potato sprouting, 419
of stem growth, 386
Inhibitors, 7, 12, 13
dormancy and, 205-6
of glucose breakdown, 276
of glutathione reductase, 318
light as, 83
in phosphorylation, 343-44
of photosynthesis, 347-48
of root elongation, 239, 240, 242-50
of seed germination, 302, 305-10
to virus inoculation, 144, 156
Insects as virus transmitters, 148-49, 166-67
Internode, 192, 198
elongation and light quality, 378, 386, 394, 395
Ions
carriers and, 7-12
growth and, 16-17
metabolism and, 12-16
passive movement, 2-7
specificity, 10-12
translocation of, 17-19
Iron
in animals, 172-73
in chloroplasts, 176-77
chlorosis, see Iron chlorosis
enzymes, 172
functions of, 172
requirement, 175-78
supply, 172-75
Iron chlorosis, 171-87
bicarbonate ion and, 178-180
causative factors, 178-87
chelates and, 184-87
copper and, 173-75
environmental factors, 183
macronutrients and, 180-182
micronutrients and, 182-183
oxygen and, 179
soil iron status and, 171, 174, 178-87
Irradiation of plants, 389-392
Isotope exchange, 8

K

Krebs cycle, 268, 283
inhibition of, 13

L

Lactones as inhibitors, 247-48
Langmuir isotherm, 9
Leaf
abscission, 194, 208
amino acid formation in, 403
calcium absorption in, 16
endogenous rhythm and, 75, 83
glucose uptake in, 333
growth of and light, 386
iron in, 176, 177, 186
light absorption in, 389
light effect on photosynthesis, 389
light reflection in, 389
light transmission in, 389
movement of and light, 380
number of and photosynthetic activity, 403

penetration of, 358
pentose phosphate cycle compounds and, 277
photoperiodism and, 198-200, 205
structure of and light, 374
virus infection and, 151-52 158, 163
virus introduction in, 144
wax on, 357
Leafhoppers, see Virus, movement of
Legumelin, 221
Legumin, 217, 221-23, 233
as enzyme, 224
Lichen, 135
Light
absorption, 44-46
chlorophyll and, 31-32, 35-37
chloroplast components and, 37-40
chloroplast multiplication and, 92
CO_2 production and, 79
endogenous rhythm and, 73-76
flashing experiments, 36-37
formative effects of, 374
germination and, 301, 304-5 308, 312-17, 374
and growth of blue-green algae, 54
incandescent and photoperiodism, 391
induced phosphorylations, 337-52
as inhibitor, 83, 381, 384
irradiation for horticultural purposes, 389-92
leaves and, 374
and nitrogen fixation, 60, 62
and pentose phosphate cycle, 278, 283
pigments and, 393-97
phosphate assimilation and, 329-30, 334, 335
photosynthetic products and, 403
protoplasm viscosity and, 393
quality and plant growth, 373-97, 404
reaction of photosynthesis, 25
and respiratory rate, 392-393
sporulation and, 74-76
viruses and, 163
wave length and plant growth, 373-97
see also Photoperiodism
Lignin, 412
Luminescence, 46-47
Lupin
globulin, 228
seed protein, 226

M

Manganese, and iron chlorosis, 183
Mannoheptulose, 288-89
Meristem
cuticle of, 356
light and, 198
virus infection of, 150, 151
Mesocotyl, inhibition of, 385, 386
Mesophyll, wetting of, 358
Metabolism
endogenous rhythm and, 77-81
ions and, 12-17
of mitosis, 102-7
Microsomes, ions in, 13
Mineral nutrition, 1-20
and flower sex, 405
Mitochondria, 93, 98, 103, 283-84, 355
in culture, 135
in germination, 300
iron and, 177
and respiratory chain phosphorylation, 338-39, 345
transport in, 13-14
Mitosis, 81, 96, 99-107
cleavage, 102
inhibition and, 109-10
metabolism and, 102-7
movement, 102
polarity, 101-2
stimulation of, 110-11
Molybdenum, and iron chlorosis, 183
Mosaic, see Viruses
Mutation, in obligate parasites, 122-24
Myxomycetes, as obligate parasites, 130

N

Naras seed proteins, 231
Nematodes in culture, 135
Nickel, and iron chlorosis, 183
Nitrate
and acid synthesis, 404
as germination promoter, 308, 309-10
nitrogen fixation and, 61
uptake, 361
Nitrogen
in carbohydrate metabolism, 277, 278
and glucose assimilation, 332-33
metabolism, 215, 401
Nitrogen fixation, 51-67
in bacteria, 52-53, 58-61
in blue-green algae, 51-52, 58-61
in green algae, 53-54
in higher plants, 53-54
light and, 60, 62
mechanism of, 60-61
photosynthesis and, 61-63
role of, ecologically, 65-67
symbioses and, 63-65
Nucleic acid, 95
synthesis of, 411
Nucleus
chromosomal multiplication and, 94-95
dormancy and, 418
iron in, 176
Nutational movement, 72
Nutrients
and root elongation, 238
transfer by dodder to host, 147
Nutrition
foliar, 405
mineral, 1-2
drought resistance and, 414

O

Obligate parasitism
adaptation, 122-24
animal comparison, 121-22
appressoria, 125
degeneration and, 128-29
degrees of, 121
definition, 115-17
evolution, 122-24
heteroecism, 128
host range, 127-28
host reaction, 126-27
hypersensitivity, 128
immunity, 128
importance, 115
intracellular, 124-25
metabolites, 129
mutation, 122-24
problem, 117-21
scope, 117
specialization, 127
taxonomic groups, 125-26, 129-35
thigmotropic response, 127
Oil, penetration of, 358
Oxygen
and auxin transport, 367
consumption in mitosis, 103-7
deficiency and oil formation, 413
and germination, 303
and iron chlorosis, 179
and phosphorylation, 335, 340
uptake, 270

P

Parasites
as virus transmitters, 148

see Obligate parasitism
Parenchyma
auxin transport in, 366
virus infection and, 151-52 157, 158-59, 166
Pea protein, 221-26, 233-34
Peanut globulins, 227-28
Pectin
in epidermis, 356
formation, 284-85
Pentose cycle, 27
Pentose phosphate pathway, 275-83
enzymes of, 269-74
Peptide, 223
Permeability, contact angle and, 357-58
Peronosporales, as obligate parasites, 130-32
Peroxidase, iron salts as, 172
Petals, endogenous rhythm and, 75, 83
pH
and auxin uptake, 360-61
blue-green algae and, 56
cytochrome oxidase and, 14
globulin solubility and, 231
and iron chlorosis, 172, 174, 178, 179-80, 185
in phosphorylation, 29
photosynthetic bacteria and, 60
root elongation and, 245-46, 251
Phloem
auxin movement in, 364-65
virus and, 147, 149, 154-55, 160-65
Phony virus of peach, 159-60
Phosphate
assimilation of, 329-32
in carbohydrate metabolism, 267
esterification of, 331-32
in photosynthesis, 325-29
transport of, 364-65
6-phosphogluconate
cleavage, 292
dehydrogenase, 272
Phosphopentokinase, 290-91
Phosphorus
and iron chlorosis, 180-82
metabolism of and photosynthesis, 325-52
early hypotheses, 325-29
"energy rich", 325-52
light induced phosphorylations, 337-52
whole cell experiments, 329-37
in obligate parasitism, 120
Phosphorylation, 326, 328, 333
light-induced, 337-52
Photochemistry, of chlorophyll, 31
Photomorphogenetic reactions, 382-85, 393-97
Photoperiodism, 374
endogenous rhythm and, 81-84
germination and, 202-4, 316-17
recent work in U.S.S.R., 421
in woody plants, 191-211
ecological importance of, 207-11
locus of, 198-200
partial processes of, 200-207
types of, 191-98
Photoreceptive systems, 393-97
Photosynthesis
bacterial, 30-34
chloroplast reactions, 43-44
CO_2 reactions in, 26-30, 289-90
dehydrogenase in, 402
energy transformations in, 25-47
fluorescence and, 46-47
free energy requirements, 41-43
gas exchange measurement, 43
at high altitude, 404
light absorption in, 44-46
light quality and, 382, 388-389
luminescence and, 46-47
nitrogen fixation and, 61-63
periodicity, 77-78
and phosphorus metabolism, 325-52
pigments and, 32-34
primary reaction, 25
products of and light quality, 403
products of and temperature, 403
rates of, 36-37
salinity and, 416
types of reaction, 25
work in U.S.S.R., 402-4
Photosynthetic organisms, nitrogen fixation in, 51-67
Phototropism, 394
Phycocyanin, 385
Phycoerythrin, in photosynthesis, 33
Pigments, 401
germination and, 315-16
light reception and, 393-397
role in photosynthesis, 32-34
Plant, woody, see Woody plants
Plant physiology, recent advances in U.S.S.R., 401-22
Plant regulators, absorption and translocation, 355-69
Plasma membrane, permeability and, 359-61
Plasmodesmata
permeability and, 359
as virus pathway, 159
Plasmolysis, 8
Plastids
endogenous rhythm and, 77
enzymes in, 409
Polar transport, of hormones, 366-68
Pollen
in mitosis, 103
as virus transmitter, 157
Polyphosphates, 336
Porphyrins, 172
Potassium, iron chlorosis and, 181
Powdery mildew, see Obligate parasitism
Protein
chromosomal duplication and, 95-96
classification of, 215-16
climate effect on, 232-33
complexes in chloroplasts, 403
endogenous rhythm and, 71-72
as enzymes, 216
formation and light quality, 403
function in permeability, 359
germination and, 233-34
iron effect on, 172, 173, 176, 181, 183
isolation experiments, 230-32
metabolism, 215
ripening and, 233-34
in root elongation, 241, 246
salinity and, 416
seed globulins as dissociating systems, 227-30
seed proteins, 216-26
serum, 216
synthesis in mitosis, 104
synthesis, and ion transport, 17
Protoplasm
drought resistance and, 414
endogenous rhythm and, 71-72
viscosity of and light quality, 393
Protoplasmic streaming, 103
auxin transport and, 368
virus infection and, 159
Protoplast, 359
Psorosis, 145
Pyruvic acid, in carbohydrate metabolism, 268
Pyruvate, aerobic consump-

tion in plants, 283-84

Q

Quantum requirement, 40-41

R

Resonance, 34
Respiration
  "anion", 8
  enzymes in, 401
  in germination, 300, 301
  inhibitors and, 13
  ions and, 13-17
  iron and, 176, 180
  iron enzymes and, 172
  in mitosis, 104-7
  oxidative enzymes and, 408
  periodicity, 77-78
  and phosphorylation, 338-43
  rate of and light quality, 392-93
  recent work in U.S.S.R., 408
  in root elongation, 246
  salinity and, 416
  of substrates of pentose phosphate pathway, 276-277, 280-83
  translocation and, 407
Riboflavin, 402
  as photoreceptor, 76
Ribose, 277
Ribose-5-phosphate, 273
Ribulose, 272
Ribulose-5-phosphate isomerase, 273
Ribulosediphosphate carboxylase, 289-90
Rickettsias, 122
Ringspot, 145, 149
Ripening, 419
  plant proteins and, 233-34
Root
  amino acid formation in, 404, 405, 411
  CO_2 uptake in, 404
  elongation of, see Root elongation
  enzymes of, 406
  frost resistance in, 417
  ion exchange in, 3, 7
  ionic translocation in, 17-19
  iron and, 179, 186
  nucleoprotein synthesis in, 405
  nutrition, 404-6
  polyploidy in tip, 94
  protein synthesis in, 405
  recent work in U.S.S.R., 404-6
  as virus host, 150, 154, 160, 163
Root elongation
  auxins and, 250-62
  inhibition of, 242-50
  measurement of, 237-42
  physiology of, 237-62, 401
Rubber synthesis, 412
Rusts, see Obligate parasitism

S

Salinity, plant resistance to, 416
Saprophytes, 122
Scopoletin, 247
Seed
  dessication of, 302
  dormancy in, 85
  germination of, 84, 86
    see also Germination
  globulins, 227-30
  photoperiodic response, 202, 206
  proteins, 216-26
  virus transmission in, 155-57
  weed, 302
Seedling
  growth, see Photoperiodism
  root measurement tests, 237
Sex
  mineral nutrition and, 405
  salinity and, 416
Shikimic acid formation, 289
Shoot, nutational movement of, 72
Sieve tubes
  of dodder and host, 147
  as virus host, 155, 161
Soil
  iron chlorosis and, 171, 174, 178-87
  nitrogen fixation and, 65-66
Soya bean proteins, 229-30
Spermatophytes, as obligate parasites, 135
Spores, 101
  endogenous rhythm and, 74
  see also Obligate parasitism
Sprays, 356, 358
Squash seed proteins, 230-31
Stem, elongation and light quality, 378, 386
Stomata, 356
  substomatal vesicles, 125
  wetting of, 358
Sucrose synthesis, 285-88
Sugar
  and obligate parasites, 120
  reducing, in germination, 300
  synthesis of, 409
  translocation of, 407
  see also Carbohydrate breakdown
Sulfur
  in chemosynthesis, 328
  and plant protein, 232-33
Sunflower seed proteins, 231-32
Symbiosis, 122
  in blue-green algae, 63-65
Synergistic effects, 12

T

Temperature
  and auxin movement, 367
  and auxin uptake, 361-62
  blue-green algae and, 56-57
  and bud dormancy, 195-97
  and endogenous diurnal rhythm, 76-77, 84
  germination and, 84, 202-4, 303, 310-12, 316
  and iron chlorosis, 183
  and leaf abscission, 194
  and light quality effect on growth, 385
  and photosynthetic products, 403
  plant resistance to 414-15
Thermoperiodism, and endogenous rhythms, 84
Thiamine, cell division and, 108
Thigmotropism, 127
Thiorhodaceae, nitrogen fixation in, 58-60
Tidal rhythms, 85
Tonoplast, ion transport and, 17
Tracheae, virus infection and, 152-53
Transaldolase, 273-74
Transamination, 410-11
Transfer, experimental agent for, 146
Transketolase, 272-73
Translocation
  of auxins, 364-66
  cytochrome oxidase and, 407
  ion, 17-19
  recent work in U.S.S.R., 406-8
Transpiration, ions and, 18-19
Transpiration stream, salinity and, 416
Transport, mineral, 3, 7-17
Trees, photoperiodism in, 191-211
Tricarboxylic acid cycle, 63
  chlorophyll synthesis and, 403
Triosephosphate dehydrogenase, 269

U

Uptake, see Mineral nutrition
Uredinales, as obligate parasites, 133-34
U.S.S.R., recent advances in plant physiology, 401-22

V

Vacuole, ions in, 13
Vessels, ion transport and, 18

Vicilin, 217, 221-23, 228, 233
  as enzyme, 224
Violet light, see Light quality
Viruses
  biological classification of, 165-67
  biological relations of, 143-67
  insect vectors of, 143, 158-65
  modes of entrance, 143-49
  movement of, 157-65
  as obligate parasites, 121, 122, 123, 126, 127, 128, 129-30
  seed transmission of, 155-157
  tissues invaded, 150-55
Vitamin K, in photosynthesis, 39, 343, 345
Vitamins
  blue-green algae and, 56
  and obligate parasites, 121
  physiological role of, 413, 414
  synthesis, 413

W

Water
  dehydrogenation of, 25
  diffusion and, 5
  germination and, 299, 304
  and iron chlorosis, 179, 183
  permeability of cuticle and, 357, 358-59
  relations in plants, 401
  see also Drought resistance
Wax, in cuticle, 355-57
Wetting agents, 361
Wheat
  globulins, 218-220
  proteins, 217
White light, see Light quality
Withering, 414, 415
Woody plants, photoperiodism in, 191-211

X

Xylan, 278, 285
Xylem
  of dodder and host, 147
  virus movement in, 159-60
  as virus recipient, 149, 152-154
Xylose, 277
Xylulose phosphate formation, 291-92

Y

Yeast, 267, 272, 281
Yellow light, see Light quality